Antibiotic mechanisms of invertebrate and mammalian defensins

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Abbreviations

A_λ	absorbance at a wavelength of λ
ABC	ATP binding cassette
ad	fill up to
AD	atopic dermatitis
AM	amidase
APS	ammonium persulfate
ATCC	American Type Culture Collection
BCA	bicinchoninic acid
BHI	brain heart infusion (complex medium)
BSA	bovine serum albumin
°C	degree Celsius
C_{55}-P	undecaprenyl phosphate (lipid carrier)
CCCP	carbonyl cyanide m-chlorophenylhydrazone
CD	Crohn´s disease
CENTA™	chromogenic cephalosporin (β-lactamase substrate)
CF	carboxyfluorescein
CFU	colony forming unit(s)
Cg-Def	defensin from the oyster *Crassostrea gigas*
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
Ci	Curie
cpm	counts per minute
CSαβ	cysteine-stabilised α-helix β-sheet (motif)
3D	three-dimensional
Da	Dalton
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
DMSZ	German Research Center for Microorganisms and Cell Culture
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
EDTA	ethylenediaminetetraacetic acid
et al.	et alii, and others
GL	glucosaminidase
GlcCer	glucosylceramide
GlcNAc	N-acetylglucosamine

h	hour
hBD	human β-defensin
HD	human defensin (α-defensin)
HDP	host defence peptide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HNP	human neutrophil peptide (α-defensin)
IC_{50}	half maximal inhibitory concentration
IFN	interferon
IL	interleukin
IPTG	isopropyl-β-D-galactopyranoside
l	liter
LB	lysogeny broth (complex medium)
lipid II	undecaprenyl-pyrophosphoryl-MurNAc-pentapeptide-GlcNAc
LPS	lipopolysaccharides
LTA	lipoteichoic acid
M	mol
MALDI-TOF MS	matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry
MES	2-(N-morpholino)ethanesulfonic acid
MIC	minimal inhibitory concentration
MilliQ	ultrapure water (type I)
min	minute
MRSA	methicillin-resistant *Staphylococcus aureus*
MurNAc	N-acetylmuramic acid
MW	molecular weight
MWCO	molecular weight cut-off
Ni-NTA	nickel-nitrilotriacetic acid
NMR	nuclear magnetic resonance
OD_{600}	optical density at a wavelength of 600 nm
ONPG	o-nitrophenyl-β-D-galactopyranoside
PBP	penicillin binding protein
pH	pondus hydrogenii, hydrogen ion concentration
PMA	phosphomolybdic acid
R	resistance
RNA	ribonucleic acid

RNase	ribonuclease
RP-HPLC	reversed-phase high performance liquid chromatography
rpm	rounds per minute
RT	room temperature
RTD	Rhesus macaque θ-defensin
RU	response unit(s)
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPB	sodium phosphate buffer
SPR	surface plasmon resonance
SUV	small unilamellar vesicle
TAE	Tris-acetate-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TLC	thin layer chromatography
TLR	Toll-like receptor
TNF	tumour necrosis factor
TPP^+	tetraphenylphosphonium bromide
Tris	tris(hydroxymethyl-)aminomethan
Triton X-100	polyethylene glycol *tert*-octylphenyl ether
TSB	tryptic soy broth (complex medium)
UDP	uridine diphosphate
UDP-MurNAc-pp	UDP-N-acetylmuramyl-pentapeptide
V	volt
VRE	vancomycin-resistant enterococci
v/v	volume per volume
w/v	weight per volume

The abbreviations of the amino acids follow the IUPAC regulations. For time, length and mass description the standard IS units were used.

1 Introduction

In 1928, Alexander Fleming discovered the first antibiotic – penicillin – that was subsequently developed for clinical use (Fleming, 1929). The effective treatment of previously deadly infectious diseases revolutionised medicine, and contributed to increasing human life expectancy.

Since that time, numerous natural and synthetic antimicrobial substances have been identified. However, the development and clinical use of each new antibiotic class was followed by emergence of resistant bacteria. Today, the therapeutic options for multidrug resistant pathogens are limited, and in the last decade only two new antibiotic classes entered the market. Consequently, there is an urgent need to discover and develop novel anti-infective agents to combat pathogenic bacteria (Brotz-Oesterhelt and Sass, 2010; Davies and Davies, 2010).

Host defence peptides, which are produced by almost all eukaryotic organisms, are considered as a promising source of potential antibiotics since they combine direct antibacterial activities with modulation of immune response. Moreover, they are also active against those bacteria resistant to conventional antibiotics and show only modest resistance development under *in vitro* selection pressure (Hancock and Sahl, 2006; Yeung et al., 2011).

1.1 Host Defence Peptides

Multicellular organisms defend themselves against invading pathogens by producing a variety of antimicrobial peptides referred to as host defence peptides (HDPs). These peptides are an essential component of the ancient, non-specific innate immune system that represents a first line of host defence. Depending on the organism and tissue type, HDP expression can be constitutive or induced in response to infection and provide either a systemic or a local protection of the host.

HDPs display direct antimicrobial activity against a broad range of microorganisms, including bacteria, fungi and even certain protozoa and enveloped viruses. The most potent peptides kill at low micromolar concentrations and some also exert activity against multidrug resistant microbes. In addition to their function as endogenous antibiotics, they exhibit various immunomodulatory activities (section 1.4).

Generally, HDPs are gene-encoded peptides and derive from larger precursors by proteolytic processing. They are short (12 to 50 amino acids), cationic (net charge ranging from +2 to +11) and are able to adopt an amphipathic structure (often in contact with membranes) in which hydrophobic and cationic amino acid residues are clustered into

distinct domains (Ganz and Lehrer, 1998; Hancock and Lehrer, 1998; Jenssen et al., 2006; Zasloff, 2002).

Over the past decades, more than one thousand of these peptides from plants, fungi, invertebrates and vertebrates have been identified, indicating the abundance of HDPs. Their sequences are compiled in three antimicrobial peptide databases (http://aps.unmc.edu/AP/main.html; http://www.bbcm.univ.trieste.it/~tossi/pag1.htm; http://phytamp.pfba-lab-tun.org/main.php). Despite a high degree of sequence variation, HDPs have been classified into four groups according to their secondary structure, amino acid composition and number of disulphide bonds:

(i) linear α-helical peptides that do not contain cysteine residues, e.g. LL-37 (human), magainin (frog; Figure 1);

(ii) cysteine-free, extended helical peptides with a predominance of one or two amino acids, such as tryptophan-rich indolicidin (cow; Figure 1) or proline-rich PR-39 (pig);

(iii) peptides with a loop structure and two disulphide-bridged cysteines; representatives are bactenecin (cow) and thanatin (insect; Figure 1);

(iv) peptides containing β-sheet elements stabilised by two to four intramolecular disulphide bonds, e.g. hBD2 (human; Figure 1).

The latter group includes defensins, a peptide family that was first discovered in mammals and subsequently found in invertebrates, plants and fungi (Hancock, 1997; Hancock and Diamond, 2000).

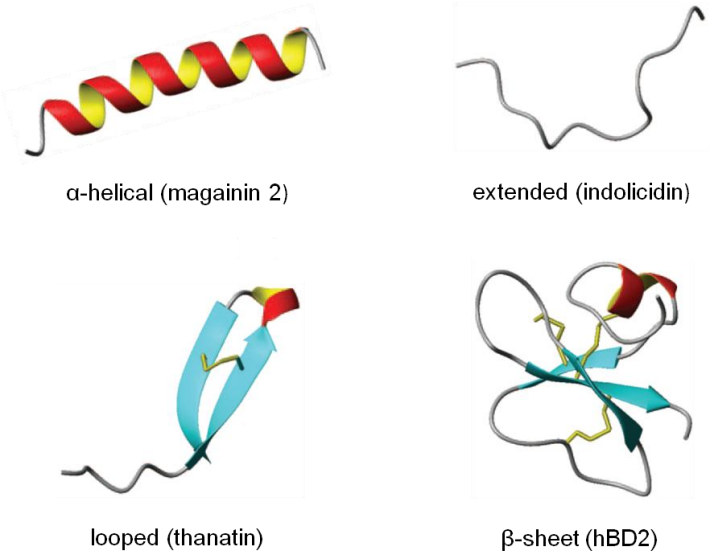

Figure 1 Structures of host defence peptides (modified according to Jenssen et al., 2006).

1.2 Defensins

Members of the defensin family can be found in three eukaryotic kingdoms – in fungi, plants and both invertebrate and vertebrate animals.

Vertebrate defensins can be subdivided into three families, α-, β- and θ-defensins, based on precursor and gene structure as well as spacing and pairing of their six conserved cysteine residues. Both α- and β-defensins are composed of a triple-stranded antiparallel β-sheet structure stabilised by three disulphide bridges (Figure 2). Alpha-defensins (disulphide pairing C1-C6, C2-C4, C3-C5) are present in high (up to millimolar) concentrations in the granules of neutrophils and small intestinal Paneth cells, whereas β-defensins (disulphide pairing C1-C5, C2-C4, C3-C6) are mainly expressed in epithelial tissues.

To date, four human neutrophilic α-defensins (HNP1-4), two human enteric α-defensins (HD5, HD6) and four human epithelial β-defensins (hBD1-4) have been characterised that differ substantially in their potency and activity spectra (Gram-positive and Gram-negative bacteria, fungi, viruses) (Ganz, 2003; Schneider et al., 2005). Interestingly, human β-defensin 1 (hBD1) becomes a potent antimicrobial peptide only after reduction of its disulphide bonds, then acting against the yeast *Candida albicans* and anaerobic Gram-positive commensals of the gut (Schroeder et al., 2011).

However, bioinformatic approaches revealed that the human genome encodes more than 30 β-defensin genes in five chromosomal regions (Schutte et al., 2002; Yamaguchi et al., 2002). The expression of many of these genes has been confirmed on mRNA level; several are specifically expressed in the testis and epididymis (Pazgier et al., 2006; Rodriguez-Jimenez et al., 2003; Yamaguchi et al., 2002), suggesting an involvement of β-defensins in sperm maturation (Zhou et al., 2004). In addition to defensins, humans synthesise the α-helical cathelicidin LL-37. Cathelicidins comprise structurally diverse HDPs whose precursors contain a conserved N-terminal cathelin domain and a C-terminal antimicrobial domain (Durr et al., 2006).

Theta-defensins have been isolated so far from the neutrophils and monocytes of several species of Old World monkeys and arose from a pre-existing α-defensin. Their peptide backbone is cyclised by ligation of two identical or similar nonapeptides and the resulting peptide ring is stabilised by six disulphide-bridged cysteines (disulphide pairing C1-C6, C2-C5, C3-C4; Figure 2) (Tang et al., 1999; Tran et al., 2002). Noteworthy, there are six θ-defensin genes present in the human genome, but a premature stop codon in the signal sequence aborts their translation, thus causing a higher susceptibility of humans towards HIV-1 infections (Nguyen et al., 2003).

Invertebrate defensins have been found in the hemolymph (plasma and hemocytes) and certain epithelial cells of arthropods (e.g. insects) and mollusks. Functionally, antibacterial (primarily active against Gram-positive bacteria) and antifungal peptides can be distinguished which differ from vertebrate defensins by their disulphide bridging patterns (C1-C4, C2-C5, C3-C6 for peptides containing six cysteines). The core structure of invertebrate defensins is composed of an α-helical domain linked to a two-stranded antiparallel β-sheet with three or four disulphide bonds forming the so-called cysteine-stabilised α-helix β-sheet (CSαβ) motif (Figure 2). Some antifungal peptides like drosomycin from *Drosophila melanogaster* contain an additional short N-terminal β-strand presenting a βαββ-scaffold that is similar to that of antifungal plant defensins (Figure 2) (Bulet et al., 2004; Thomma et al., 2002). Meanwhile, peptides of another defensin family referred to as “big defensins” have been isolated from invertebrates (Gerdol et al., 2011; Rosa et al., 2011; Saito et al., 1995; Teng et al., 2012; Zhao et al., 2007). They consist of 79-94 amino acids that form two distinct structural and functional domains. The hydrophobic N-terminus exhibits selective activity against Gram-positive bacteria, while the hydrophilic C-terminus displays Gram-negative activity. Interestingly, the C-terminal region adopts the typical fold of β-defensins with identical disulphide arrays (C1-C5, C2-C4, C3-C6; Figure 2), suggesting that β-defensins emerged from an ancestral big defensin (Zhu and Gao, 2012).

Defensins with a high degree of sequence and structural similarity to invertebrate defensins have been identified in several fungi (Mygind et al., 2005; Zhu, 2008). For example, plectasin isolated from the saprophytic ascomycete *Pseudoplectania nigrella* is characterised by a CSαβ motif (disulphide pairing C1-C4, C2-C5, C3-C6; Figure 2) and displays potent antibacterial activity (Mygind et al., 2005).

All plant defensins known so far are characterised by four disulphide bridges and share the same cysteine pairing pattern (C1-C8, C2-C5, C3-C6, C4-C7; Figure 2). They are mainly active against fungi, including plant and human pathogens (Tavares et al., 2008; Thevissen et al., 2007). In past years, increasing evidence for other biological activities has been gathered such as inhibition of α-amylase activity and protein synthesis, antiviral and antitumour activity, inhibition of plant root growth, blocking of ion channels and mediation of zinc tolerance in plants (Aerts et al., 2008; Allen et al., 2008; Lin et al., 2009; Mirouze et al., 2006; Spelbrink et al., 2004; Wong and Ng, 2005).

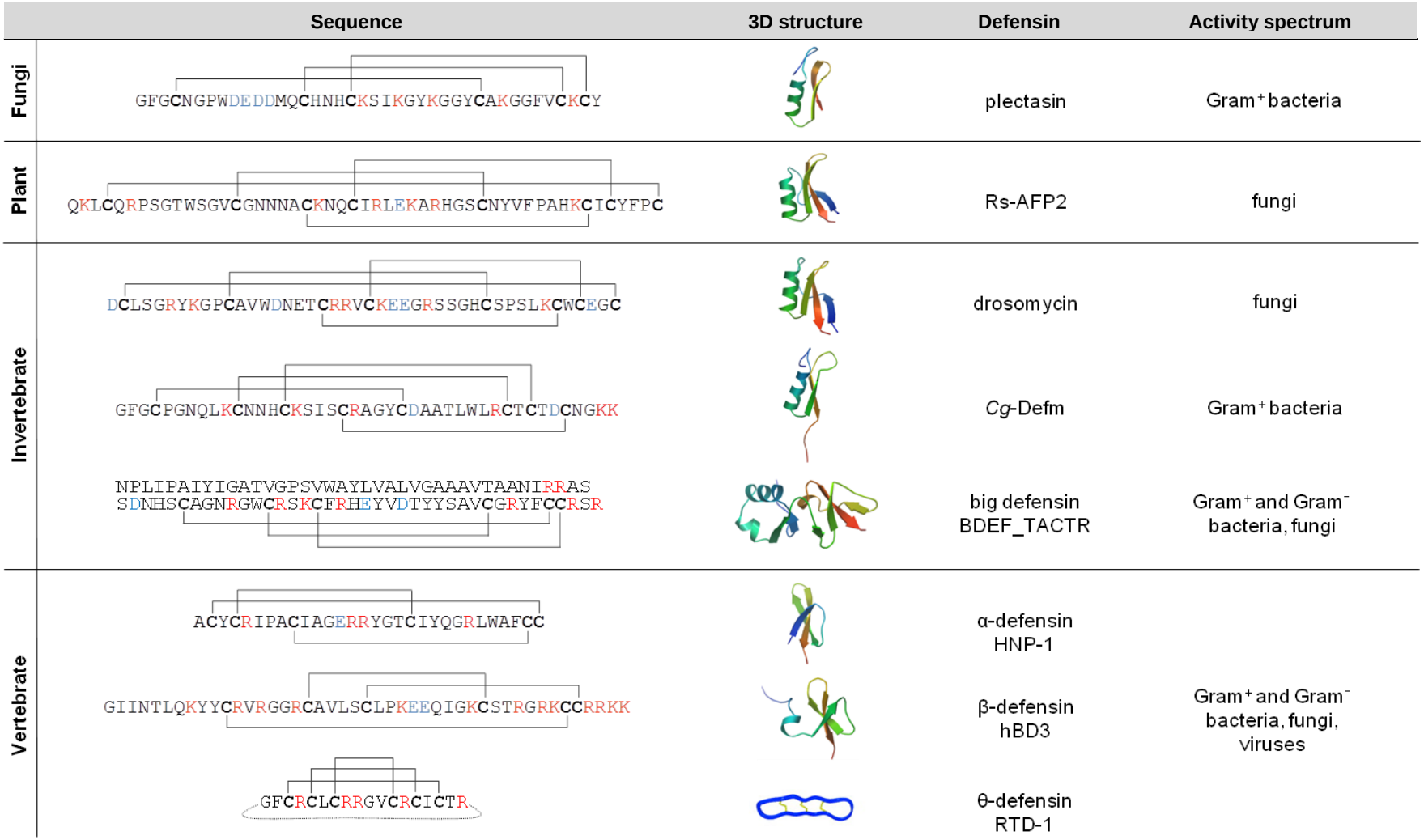

Figure 2 Sequences and 3D structures of selected defensins. Residues with a positive charge are marked in red, negatively charged residues are marked in blue. The 3D structure of RTD-1 was taken from Trabi et al., 2009; all other 3D structures were obtained using Swiss Model (www.swissmodel.expasy.org).

It has been postulated that all defensins evolved from a single precursor, based on (i) sequence and structural similarity of plant and insect defensins as well as fungal and antibacterial invertebrate defensins, (ii) similar mode of action of plant and insect defensins (section 1.3) and (iii) inter-functionality of defensins from different kingdoms (Thevissen et al., 2004; Zhu and Gao, 2012). In this respect, overexpression of insect (Langen et al., 2006) or mammalian defensins (Aerts et al., 2007) in plants resulted in increased plant resistance to fungal diseases, comparable to that obtained by overexpression of plant defensins. Vice versa, intravenous injection of plant defensins into *C. albicans* infected mice resulted in a significant reduction of the fungal burden in the kidneys of the infected mice at least as efficiently as the standard drug fluconazole (Tavares et al., 2008). In line with these observations, Yount and Yeaman (2004) identified a conserved three-dimensional structural motif in disulphide-containing host defence peptides from virtually all organisms, the so-called γ-core motif (Figure 3).

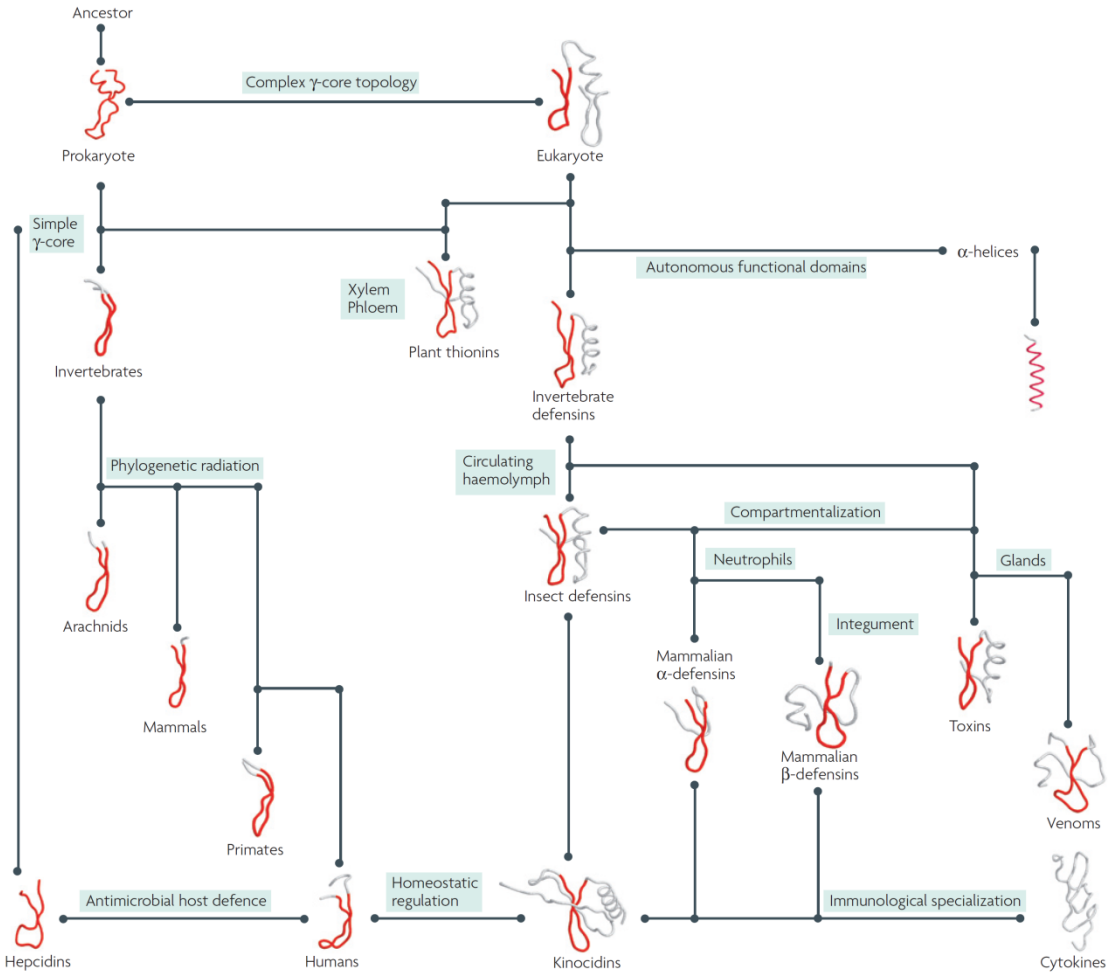

Figure 3 The common ancestor hypothesis of disulphide-stabilised host defence effector molecules. The γ-core structural motiv conferring antimicrobial activity has been conserved during evolution and can be traced back to prokaryotic origin (Yeaman and Yount, 2007).

This motif arises from the bidirectional orientation of a specific amino acid sequence and is composed of two antiparallel β-sheets with an interposed short turn region. Indeed, this motif can also be found in other cysteine-stabilised host defence effector molecules with antimicrobial activity like venoms, toxins or microbicidal chemokines. Thus, it is proposed that disulphide-containing HDPs emerged from a common ancestral peptide that can be traced back to prokaryotic origin (Figure 3) (Yeaman and Yount, 2007).
Consistent with this hypothesis, two defensin-like peptides (DLP) were recently identified in the myxobacteria *Anaeromyxobacter dehalogenans* and *Stigmatella aurantiaca*. Despite the lack of two cysteine residues, the *A. dehalogenans* DLP exhibits the typical CSαβ fold of fungal, invertebrate and plant defensins and displays activity against the malaria parasite *Plasmodium falciparum* (Gao et al., 2009; Zhu, 2007).

1.3 Antimicrobial mode of action of HDPs

Almost all HDPs are cationic and amphipathic. Hence, it has been frequently demonstrated, using model membranes and unilamellar vesicles of various lipid compositions, that HDPs interact with negatively charged components of the microbial surface and subsequently disrupt membrane barrier function via pore-formation or unspecific membrane permeabilisation (Gazit et al., 1995; Henzler-Wildman et al., 2004; Yang et al., 1998; Yang et al., 2001). Numerous reports on biophysical behaviour of synthetic and natural peptides in lipid bilayers were published and different models have been elaborated to describe the membrane-peptide interaction. The best studied peptides among HDPs are linear peptides that are unstructured in solution and adopt an α-helical conformation in the presence of membranes.
In this context, it was reasoned that positively charged cationic peptides are electrostatically attracted to negatively charged phospholipids which are typically present in the outer leaflet of microbial membranes. With increasing peptide concentration the peptide molecules insert into the bilayer either forming transmembrane pores or disrupting the membrane in a detergent-like manner (Brogden, 2005; Zasloff, 2002). For example, the formation of “toroidal pores” was demonstrated for magainin 2 (Yang et al., 1998) and LL-37 (Henzler-Wildman et al., 2004), whereas the induction of “barrel-stave” pores seems unique for the non-ribosomal peptide antibiotic alamethicin (Yang et al., 2001). Alternatively, peptides such as cecropins accumulate parallel to the membrane surface and at sufficient high concentration cause disintegration of the lipid bilayer (“carpet model”) (Gazit et al., 1995; Zasloff, 2002). In all model systems, membrane permeabilisation depends on the lipid composition (chain length and charge of phospholipids). The interaction of peptides is strongly reduced by zwitterionic

phospholipids or cholesterol, both prominent constituents of eukaryotic cell membranes - a feature that might explain the reduced toxicity towards host cells.

Hence, in the past, discussions on the mode of microbicidal action of HDPs mainly focused on the lipid bilayer as a target, whereas membrane proteins making up 50% or more of microbial membranes were hardly considered. While the relevance of the lipid interactions for the attraction of HDPs to the membrane interface can hardly be questioned, the relevance for the actual killing process may be overestimated. There is increasing evidence that some HDPs enter the cytoplasm without disrupting the membrane bilayer; once inside the cell, they may interfere with nucleic acid and/or protein synthesis (Boman et al., 1993; Lehrer et al., 1989; Park et al., 1998; Patrzykat et al., 2002; Subbalakshmi and Sitaram, 1998). Moreover, HDPs have to cross barriers such as cell wall peptidoglycan or the outer membrane of Gram-negative bacteria to interact with the cytoplasmic membrane. For Gram-negative bacteria a translocation mechanism termed “self-promoted uptake” has been described. Thereby, the peptides first bind to the lipopolysaccharides (LPS) of the outer membrane and subsequently cause displacement of divalent cations (Mg^{2+}, Ca^{2+}) that bridge and neutralise LPS. Thus, destabilised outer membrane areas are formed through which peptides can translocate (Hancock, 1997).

Recently, it has become evident that the fungal defensin plectasin (Schneider et al., 2010), the α-defensin human neutrophil peptide 1 (HNP-1) (de Leeuw et al., 2010) and the human β-defensin 3 (hBD3) (Sass et al., 2010) specifically inhibit bacterial cell wall biosynthesis by targeting lipid II (Figure 27). Plectasin exhibits potent activity against several Gram-positive bacteria. It does not compromise membrane integrity at all, since no impact on membrane potential and on intracellular K^+ contents could be observed with cells exposed to plectasin concentrations in the MIC range. Instead, *in vitro* and *in vivo* experiments demonstrated that plectasin forms a stoichiometric complex with the cell wall precursor lipid II in a 1:1 molar ratio, thus making it unavailable for the interaction with cell wall biosynthetic enzymes. NMR-based modelling of the plectasin-lipid II complex indicated that the defensin interacts with the pyrophosphate moiety of lipid II via hydrogen bonding, whereas the hydrophobic part of the peptide is located on the membrane surface. Additionally, a salt bridge between the N-terminus (H18) and the D-glutamic acid in position 2 of the stem peptide is crucial for binding (Figure 4) (Schneider et al., 2010).

The killing of *S. aureus* by hBD3 is the result of pleiotropic effects. Beside lipid II sequestration, the peptide seems to have more generalised effects on membrane bound processes such as the electron transport chain (Sass et al., 2008; Sass et al., 2010). De Leeuw et al. (2010) proposed that the antimicrobial action of HNP-1 is also based on lipid II binding as the ability of the peptide to kill *S. aureus* is strongly reduced in cells with altered lipid II levels. Moreover, they showed that the affinity towards lipid II is significantly

higher for the natural peptide than for a synthetic peptide composed entirely of D-amino acids.

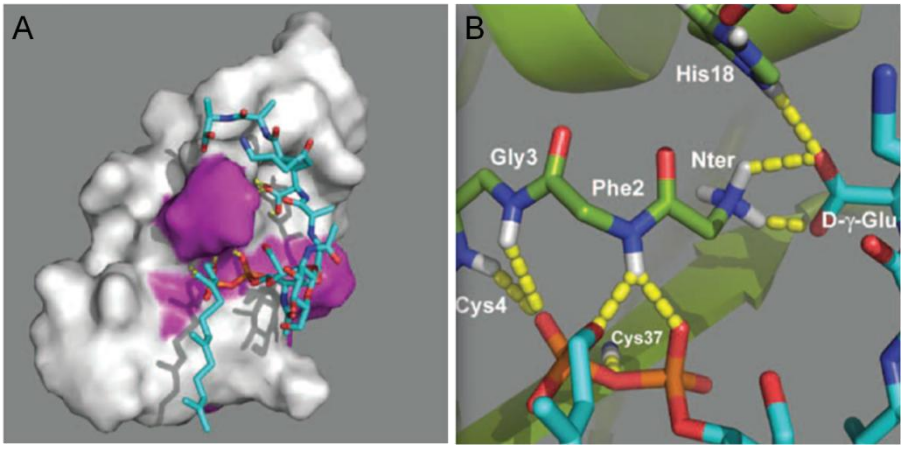

Figure 4 A: NMR-based modelling of the plectasin-lipid II complex. B: The fungal defensin interacts with the pyrophosphate moiety of lipid II by forming four hydrogen bonds (involving residues F2, G3, C4 and C37). Moreover, the D-glutamate of lipid II forms a salt bridge with the N-terminus and the H18 side-chain of plectasin (Schneider et al., 2010).

Additional antibacterial effects could be observed for LL-37 and indolicidin. At subinhibitory concentrations, both peptides prevent potently the biofilm formation of *Pseudomonas aeruginosa* and even induce the dissolution of existing biofilms. It has been shown that LL-37 acts in three ways: it reduces the attachment of cells to the surface, stimulates the surface motility of cells and affects the two major quorum sensing systems – Las and RhI – resulting in downregulation of essential biofilm-related genes (Overhage et al., 2008).

The antifungal action of plant and insect defensins is based on interaction with particular sphingolipids in membranes and cell walls of susceptible fungi. Sphingolipids are an important structural component of eukaryotic membranes and are also recognised as secondary messenger molecules regulating the equilibrium between cell death and cell growth processes (Thevissen et al., 2006). The interaction of the plant defensin Rs-AFP2 (*Raphanus sativus* antifungal protein 2) with glucosylceramides (GlcCer) in the fungal membrane modulates cellular processes such as apoptosis, thereby leading to fungal cell death (Aerts et al., 2009; Thevissen et al., 2007). Moreover, it has been demonstrated that binding of Rs-AFP2 to GlcCer in the cell wall of *C. albicans* causes cell wall stress, septin mislocalisation and ceramide accumulation (Thevissen et al., 2012).
The interaction with GlcCer has also been reported for Psd1 (from pea seeds) (de Medeiros et al., 2010), Sd5 (from *Saccharum officinarum*) (De-Paula et al., 2008), MsDef1 (from *Medicago sativa*) (Ramamoorthy et al., 2007) and the insect defensin heliomicin

(from *Heliothis virescens*) (Thevissen et al., 2004). Other plant defensins such as DmAMP1 (from *Dahlia merckii*) bind specifically to inositol phosphoryl-containing sphingolipids leading to membrane permeabilisation and ion efflux (Thevissen et al., 2003; Thevissen et al., 1996).

Some HDPs also exhibit antiviral activity by targeting the viral envelope directly, the viral adsorption and entry into the cell or the intracellular viral life cycle (Ding et al., 2009; Jenssen et al., 2006). Rhesus macaque θ-defensins (RTDs) and retrocyclins (synthetic θ-defensins encoded by human pseudogenes) inhibit HIV-1 attachment and entry by binding specifically to both the viral envelope glycoproteins gp120 or gp41 and the host cell receptor CD4 (Cole et al., 2002; Gallo et al., 2006; Munk et al., 2003; Seidel et al., 2010; Wang et al., 2004). The α-defensins HNP1-3 seem to have a direct effect on several enveloped viruses such as herpes simplex virus type 1 and 2 or influenza A virus, as the virions lose their ability to infect target cells after incubation with the peptides (Daher et al., 1986). Moreover, HNP1-3 inhibit multiple steps of the HIV life cycle, e.g. nuclear import and HIV replication (Chang et al., 2005; Furci et al., 2007; Mackewicz et al., 2003; Seidel et al., 2010; Wang et al., 2004). Antiviral properties have also been reported for β-defensins. For example, hBD2 does not only inactivate HIV particles directly, but also inhibits HIV replication (Seidel et al., 2010; Sun et al., 2005).

1.4 Immunomodulatory functions of HDPs

Research during the last years has demonstrated that HDPs do not only have direct antimicrobial activities, but also display a diverse range of immunomodulatory functions (Figure 5). As the antibacterial properties of several HDPs are strongly antagonised under physiological salt concentrations or serum components, it has been proposed that the immunomodulatory activities of some of these peptides may be more important to mediate bacterial clearance *in vivo*.

Mammalian HPDs are expressed either constitutively or are inducible in various cell types such as neutrophils, monocytes/macrophages and epithelial cells. The expression of human β-defensins can be triggered by conserved bacterial structures (e.g. LPS) via Toll-like receptors (TLR) or by proinflammatory stimuli (e.g. TNF-α, IL-1β or IFN-γ). In contrast, α-defensins are expressed constitutively in the bone marrow and intestinal Paneth cells, but their secretion can be stimulated, e.g. by bacterial antigens such as OmpA or flagellin (Hancock and Scott, 2000; Selsted and Ouellette, 2005).

It has been revealed that HDPs can be chemotactic for various immune cells or they can induce the production and release of particular cytokines/chemokines, thereby recruiting effector cells to the site of infection (Chertov et al., 1996; Territo et al., 1989; Yang et al.,

2000; Yang et al., 1999). For example, hBD1 and hBD2 are chemotactic for memory T cells and immature dendritic cells by binding directly to the chemokine receptor CCR6 (Figure 5) (Yang et al., 1999), thus providing an important link between innate and adaptive immune response. HNP1-3 can attract monocytes, immature dendritic cells and $CD4^+$ and $CD8^+$ T cells (Territo et al., 1989; Yang et al., 2000). Moreover, they can induce the release of proinflammatory cytokines such as IFN-λ, IL-6 and IL-10 from T cells and TNF-α and IL-1β from monocytes (Froy, 2005; Lehrer and Ganz, 2002). The release of TNF-α and IFN-γ from macrophages stimulates in an autocrine loop the activity of phagocytic macrophages, and thereby enhances clearance of opsonised bacteria *in vitro* and in a murine model (Soehnlein et al., 2008). Additionally, several HDPs are involved in the production and release of histamine from mast cells (Niyonsaba et al., 2001).

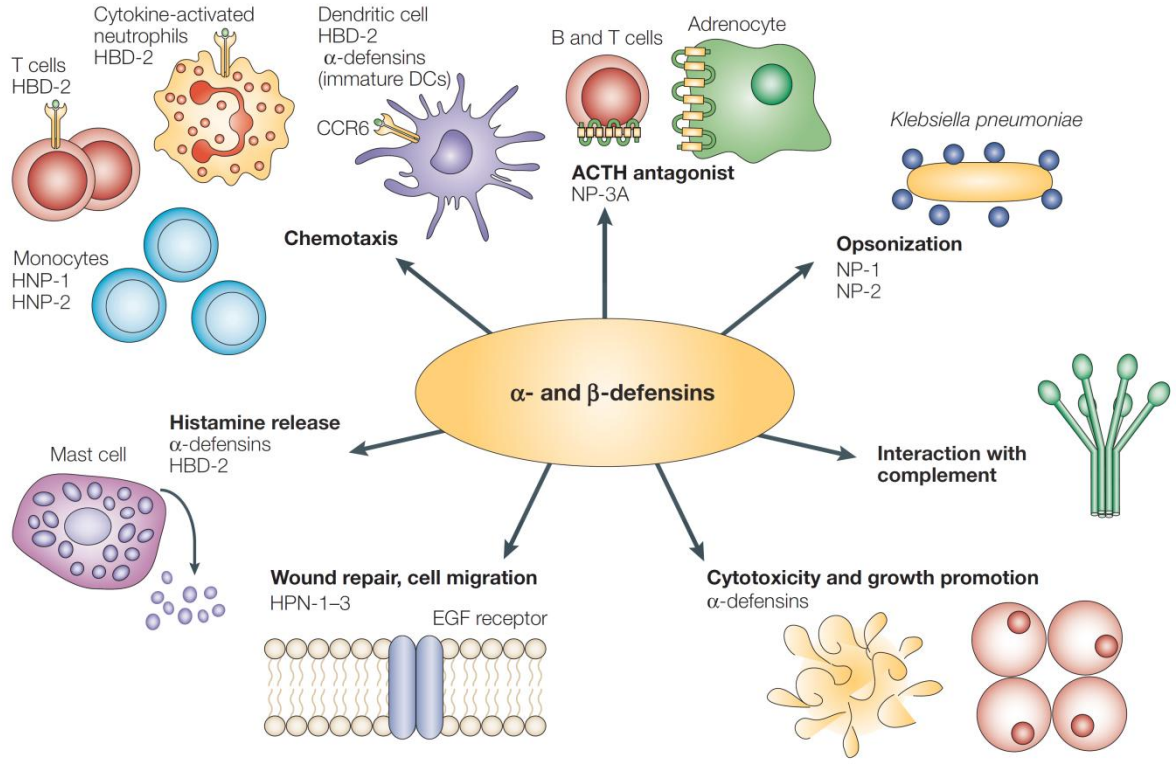

Figure 5 Immunomodulatory activities of vertebrate defensins (Lehrer, 2004).

On the other hand, HDPs can dampen harmful proinflammatory immune responses. The release of bacterial products such as LPS of Gram-negative bacteria can result in excessive inflammatory responses which lead to sepsis or related syndromes. Many peptides can limit the production of sepsis-mediating proinflammatory cytokines in TLR-4-stimulated macrophages (Scott et al., 2000; Semple et al., 2011), neutralise extracellular LPS (Mookherjee et al., 2007) and/or stimulate the expression of anti-inflammatory genes (Nijnik et al., 2009).

Further, HDPs are involved in wound healing. HBD2 and hBD3 are highly expressed in epidermal keratinocytes in response to injury or infection of the skin. They increase keratinocyte migration and proliferation through epidermal growth factor receptor signalling (Niyonsaba et al., 2007). HNP1-3 induce cell proliferation and wound closure as well as increased cell migration in the airway epithelia (Aarbiou et al., 2002; Aarbiou et al., 2004).

In addition, defensins can neutralise toxins secreted by bacterial pathogens, thereby inhibiting their cytocidal activity. It has been shown that HNP1-3 inhibit the function of lethal toxin from *Bacillus anthracis in vitro* and *in vivo* as well as of diphtheria toxin from *Corynebacterium diphtheriae* and *P. aeruginosa* endotoxin A (Kim et al., 2005; Kim et al., 2006).

The increasing knowledge about the immunomodulatory functions of HDPs led to the development of synthetic innate defence regulators (IDRs). IDRs are small, synthetic peptides derived from natural HDP templates which lack direct antimicrobial activity. IDR-1, whose sequence is based on bovine bactenecin, confers protection in a broad range of mice infection models, e.g. against MRSA, VRE and Salmonella. It selectively enhances immunity of the host by stimulating chemokine production by monocytes while suppressing potentially harmful excessive inflammatory responses (Scott et al., 2007).

1.5 HDP expression and human disorders

The gene copy number or the dysregulated expression of certain HDPs affects the pathogenesis of several infectious and inflammatory diseases, underlining the importance of these peptides in combating bacterial pathogens.

Crohn´s disease (CD) is a chronic inflammatory bowel disease that most commonly affects the ileum of the small intestine and the colon. The disease is in part attributed to the colonising bacteria of the intestine that trigger mucosal inflammation. It has been demonstrated that patients with ileal CD have a reduced expression of the intestinal Paneth cell α-defensins HD5 and HD6, suggesting that α-defensins play a role in controlling the progress of CD pathogenesis. Consistently, changes in the intestinal microbiota of transgenic mice producing HD5 in intestinal Paneth cells have been observed (Wehkamp et al., 2005). The detailed analysis of the intestinal flora of HD5-expressing mice and mice lacking Paneth cell α-defensins (due to a knockout of the processing enzyme MMP-7) clearly revealed α-defensin dependent alterations in the microbial composition, but not in the bacterial number. Thus, the deficiency of α-defensins in ileal CD may cause an imbalance of the microbial composition which in turn triggers infection and inflammation (Salzman et al., 2010). The lack of intestinal α-defensins has been linked to a diminished expression of the transcription factor Tcf-4 of the Wnt

signalling pathway which regulates differentiation of Paneth cells and α-defensin expression (Wehkamp et al., 2007).

In contrast, colonic CD seems to be associated with a low copy number of β-defensin genes encoded in a cluster on chromosome 8 (Fahlgren et al., 2004; Wehkamp et al., 2003). Fellermann et al. (2006) reported that a copy number of DEFB4 (encoding hBD2) less than four predispose to colonic CD.

Another HDP deficiency that correlates with high susceptibility to bacterial infections is Morbus Kostmann. Patients with this syndrome suffer from severe and frequent periodontal infections due to a lack of LL-37 and HNP1-3 (Putsep et al., 2002). Similarly, patients with a specific granule deficiency lacking neutrophilic α-defensins have an enhanced susceptibility to bacterial infections (Ganz et al., 1988).

Moreover, cystic fibrosis (CF) has been associated with a lack of antimicrobial activity of HDPs. This disorder is caused by a mutation in a chloride channel (cystic fibrosis transmembrane conductance regulator) regulating the chloride and sodium movement across epithelial membranes. CF is characterised by persistent colonisation of the airway epithelia, in particular by *P. aeruginosa*, inducing chronic infection and inflammation (Gadsby et al., 2006). It has been proposed that the elevated salt concentrations in the airway fluid of CF patients antagonise HDPs (LL-37 and β-defensins), thereby allowing microbial colonisation (Guggino, 2001; Smith et al., 1996). Other studies suggested that hBD2 and hBD3 are proteolytically degraded by the cysteine proteases cathepsin B, L and S present in bronchoalveolar lavage (Taggart et al., 2003), and that the reduced antibacterial activity of LL-37 is a result of direct interaction of the peptide with DNA and filamentous (F)-actin as well as released LPS found in CF sputum (Bucki et al., 2007). So far, it is unclear which of the described mechanisms plays a role *in vivo* or if they act in combination.

Moreover, HDPs protect the skin from bacterial infections and altered HDP expression levels have been related to various skin disorders. For example, patients with the inflammatory skin disease psoriasis rarely develop skin infections due to elevated HDP expression (Harder et al., 2007). In contrast, it has been assumed that the impaired induction of HDPs in the skin of patients with atopic dermatitis (AD) contribute to colonisation and infection with *S. aureus* (de Jongh et al., 2005; Nomura et al., 2003; Ong et al., 2002). Meanwhile, a study demonstrated that the expression of several HDPs is significantly induced in lesional skin of AD patients in comparison to healthy controls (Harder et al., 2010). Thus, further analysis is needed to elucidate the role of HDPs in AD.

1.6 Resistance towards HDPs

Despite the fact that HDPs are ancient molecules, they have retained their potent antimicrobial activity throughout evolution. However, some bacterial pathogens evolved mechanisms to reduce their susceptibility towards HDPs.

One of these countermeasures is based on the covalent modification of anionic cell envelope molecules, thus decreasing their net negative charge and their attraction to HDPs. The bifunctional protein MprF (multiple peptide resistance factor) of *S. aureus* catalyses the modification of the phospholipid phosphatidylglycerol with L-lysine and its translocation to the outer leaflet of the cytoplasmic membrane (Ernst et al., 2009; Peschel et al., 2001). Moreover, the anionic properties of teichoic acids, which are either anchored to membrane phospholipids or connected to peptidoglycan, are partially neutralised by D-alanylation. This reaction is catalysed by the products of the *dltABCD* operon (D-alanyl-lipoteichoic acid) (Peschel et al., 1999). Homologues of both MprF and DltABCD can be found in many pathogens (Ernst and Peschel, 2011; Peschel et al., 1999).

In *S. aureus* and *S. epidermidis*, the expression of *mprF* and *dltABCD* is controlled by the two-component regulatory system GraRS (glycopeptide resistance associated; or also known as ApsRS, antimicrobial peptide sensor). It has been proposed that the histidine kinase GraS/ApsS senses HDPs via a short extracellular loop with a high density of negative charges (Li et al., 2007a; Li et al., 2007b). Moreover, the ABC-transporter VraFG, encoded in the same operon as *graRS*, seems to be crucial for sensing of antimicrobial peptides (Falord et al., 2012). Consequently, mutations in one of the genes involved in HDP sensing or cell envelope modification significantly enhance the killing by cationic antimicrobial peptides (Kristian et al., 2003; Li et al., 2007a; Peschel et al., 1999; Sass and Bierbaum, 2009).

The two-component regulatory system PhoPQ, found in many Gram-negative species, induces the transcription of genes promoting resistance to HDPs and other cationic antimicrobial peptides, bacterial survival within macrophages and virulence. For example, the incorporation of an additional fatty acid into the lipid A portion of LPS changes the hydrophobicity of the outer membrane and reduces its permeability. Moreover, PhoPQ can activate a second two-component regulatory system, PmrAB (polymyxin resistance) which regulates the addition of aminoarabinose or ethanolamine to lipid A, thereby lowering its net negative charge (Gunn, 2008; Richards et al., 2010).

Recently, another mechanism has been described that reduces the affinity to lipid II-targeting defensins. The GatD/MurT enzyme complex of several Gram-positive pathogens is responsible for the amidation of D-glutamic acid at position 2 of the lipid II stem peptide

(compare section 1.3). Antisense-mediated depletion of GatD/MurT significantly increases the susceptibility to the fungal defensin plectasin (Munch et al., 2012).

Another strategy to escape killing by HDPs relies on the proteolytic degradation of the peptides. HDPs with an α-helical structure like LL-37 can be easily cleaved by proteases such as the metalloprotease ZapA from *Proteus mirabilis (*Belas et al., 2004*),* aureolysin from *S. aureus* (Sieprawska-Lupa et al., 2004) or the outer membrane protease PgtE from *Salmonella enterica* serovar Typhimurium (Guina et al., 2000). Some bacteria remove HDPs from their site of action by active efflux. The energy-dependent efflux pump MtrCDE (multiple transferable resistance) of *Neisseria gonorrhoeae* confers resistance to structurally diverse antimicrobial agents, including HDPs (Shafer et al., 1998).

However, all these mechanisms described above do not lead to high-level resistance. Molecules such as lipid II, LTA or LPS are formed by multistep biosynthetic processes and cannot be easily replaced or even modified without major genetic events and rearrangements. For example, the lipid II isoprenoid anchor C_{55}-P is also involved in the biosynthesis of other major cell envelope polymers, e.g. wall teichoic acid and capsules. Synthesis of C_{55}-P-anchored molecules always starts with the transfer of a sugar moiety to the lipid carrier, forming a pyrophosphate linkage. Thus, this structural motif is highly conserved as it is part of several essential building blocks. Moreover, in the host bacteria may be confronted with a mixture of HDPs that work effectively in combination and some HDPs (e.g hBD3) seem to act by attacking multiple targets.

1.7 Objectives of this work

In times in which antibiotics are becoming increasingly ineffective, there is a need to search for new therapeutic agents. HDPs, ubiquitous in nature, are promising candidates for the development of novel anti-infective drugs since they combine direct antimicrobial activity with immunomodulatory functions. However, for systematic exploitation of this concept, we need to know more about both the molecular mechanisms underlying the immune modulation and the defined antibiotic activities of HDPs.

Previous work has demonstrated that the antibacterial action of defensins can be much more targeted as it was originally proposed (compare section 1.3).

This study was designed to gain further insights into the antibacterial mode of action of different defensin groups. For this purpose, the invertebrate defensin mechanism of action was analysed on the molecular level. Many invertebrate defensins show high sequence and structural similarity to the fungal defensin plectasin, raising the question if this correlates with a similar antibiotic action. Therefore, the interaction of three oyster defensin variants with the cell wall precursor lipid II was studied *in vitro* and *in vivo*.

Moreover, vertebrate θ-defensins were included in this study to investigate their mode of action against staphylococci. Interestingly, these peptides differ structurally from all other defensins since they consist of only 18 amino acids and their backbones are cyclised by peptide bonds. It should be found out if they also affect cell wall biosynthesis or if they target other cell envelope molecules.

Since vertebrate defensins display – in comparison to plectasin – a broader activity spectrum and a lower lipid II affinity, the attraction to additional targets of Gram-negative bacteria was investigated. Hence, the interaction of hBD3 with the Gram-negative cell envelope was studied, including permeabilisation of the outer and inner membrane and susceptibility tests of *E. coli* mutants with different LPS structures.

2 Material and Methods

2.1 Chemicals and solvents

Table 1 Chemicals and solvents used in this study.

Manufacturer	Chemical/Solvent
AppliChem, Darmstadt, Germany	TEMED
Avanti Polar Lipids, Alabaster, USA	DOPC
Becton Dickinson GmbH, Heidelberg, Germany	Agar, Bacto™ tryptone, Columbia agar with 5% sheep blood, Mueller-Hinton II Agar
Bio-Rad, München, Germany	Protein assay dye reagent concentrate
Calbiochem®; Merck, Darmstadt, Germany	CENTA™ β-lactamase substrate
Fermentas GmbH, St. Leon-Rot, Germany	BSA standard set; GeneJET™ Plasmid Miniprep Kit, IPTG, Page Blue™ Protein Staining Solution, Page Ruler™ Protein Ladder (unstained, prestained), TopVision™ Agarose
GE Healthcare, Freiburg, Germany	HBS buffer (10x), NaOH
Hartmann Analytic GmbH, Braunschweig, Germany	L-[^{14}C]isoleucine, 2-[^{14}C]thymidine, [^{3}H]tetra-phenylphosphonium bromide, 5-[^{3}H]uridine
Invitrogen™, Darmstadt, Germany	NuPAGE® LDS sample buffer (4x)
Larodan Fine Chemicals, Malmö, Sweden	C_{55}-P
Leica Microsystems, Bensheim, Germany	Ultrostain 1H
Oxoid, Wesel, Germany	BHI, Mueller-Hinton broth, TSB, yeast extract
Peqlab Biotechnologie, Erlangen, Germany	PeqGold DNA ladder mix
Pierce; Distributor: Thermo Fisher Scientific, Schwerte, Germany	BCA™ Protein Assay Kit
Qiagen, Hilden, Germany	Ni-NTA-Agarose
Science Services, München, Germany	EMbed 812 Embedding Kit, Kodak D-19 developer, Agefix fixative solution
Serva Electrophoresis GmbH, Heidelberg, Germany	Acrylamide/bisacrylamide solution (19:1), 40% (w/v), anthrone
Sigma-Aldrich, Taufkirchen, Germany	Ammonium citrate, 6-aza-2-thiothymine, ammonium hydroxide, ascorbic acid, carboxyfluorescein, CHAPS, choline chloride, DNase I, ethidium bromide, glutaraldehyde (grade I, 70%), lead(II) citrate tribasic trihydrate, lipoteichoic acid (from *S. aureus*), lysozyme, MES, sephadex G-50, octyl-sepharose CL 4B, ONPG, osmium tetroxide solution (4%), pyridine, RNase A, Triton X-100, UDP-GlcNAc
Zinsser Analytic, Frankfurt, Germany	Filtersafe

All other chemicals and reagents not listed in this table were purchased from Merck (Darmstadt, Germany).

2.2 Antibiotics and antimicrobial peptides

Table 2 Antibiotics and antimicrobial peptides used in this study.

Antibiotic/Peptide	Solvent	Source
Ampicillin	MilliQ water	Sigma-Aldrich, Taufkirchen, Germany
Cg-Defs	MilliQ water	Paulina Schmitt, University of Montpellier, France
Chloramphenicol	Ethanol	Calbiochem®; Merck, Darmstadt, Germany
Ciprofloxacin (Ciprobay® 200)	Infusion solution (ready-to-use)	Bayer Healthcare, Leverkusen, Germany
Erythromycin	Ethanol	Calbiochem®; Merck, Darmstadt, Germany
hBD3	MilliQ water	Alessandro Tossi, University of Trieste, Italy
Kanamycin	MilliQ water	Sigma-Aldrich, Taufkirchen, Germany
Nisin	0.05% Acetic acid	Michaele Josten, University of Bonn, Germany
Penicillin G	MilliQ water	Sigma-Aldrich, Taufkirchen, Germany
Pep5	MilliQ water	Michaele Josten, University of Bonn, Germany
Polymyxin B	MilliQ water	Sigma-Aldrich, Taufkirchen, Germany
Rifampicin	MilliQ water	Sigma-Aldrich, Taufkirchen, Germany
RTD-1, RTD-2	MilliQ water	Michael Selsted, University of Southern California, USA
Spectinomycin	MilliQ water	Sigma-Aldrich, Taufkirchen, Germany
Tetracycline	Methanol	Sigma-Aldrich, Taufkirchen, Germany
Vancomycin	MilliQ water	Ratiopharm, Ulm, Germany

2.3 Microbiological methods

2.3.1 Bacterial strains, culture media and growth conditions

All bacterial strains used in this thesis are listed in Table 3. For long-term storage, glycerol stocks of the bacteria were prepared. Therefore, an overnight culture was mixed 1:1 with sterile glycerol (v/v) and stored in cryovials at -70°C. Strains were recovered from glycerol stocks as required by streaking out on appropriate agar plates and subsequent overnight incubation at 37°C.

Unless otherwise indicated, the cultivation of liquid cultures was performed aerobically at 37°C on an orbital shaker (170 rpm; TR-250; Infors-HF, Bottmingen, Switzerland) using a 2% inoculum (v/v) from an overnight culture. Growth media used for bacterial cultivation are denoted in Table 4.

Selective media were supplemented with antibiotics in the following concentrations: ampicillin (40 µg/ml), erythromycin (25 µg/ml), kanamycin (40 µg/ml), spectinomycin (25 µg/ml) and tetracycline (25 µg/ml).

Staphylococcus carnosus TM300 harbouring the pAH-PepI vector was grown in presence of 1% xylose to induce *pepI* expression.

Table 3 Bacterial strains and plasmids used in this thesis.

Strain or plasmid	Relevant characteristic(s)	Reference/Source
Gram-positive bacteria		
Staphylococcus aureus		
SG511-Berlin	Mutation in gene *graS*	Sass and Bierbaum, 2009
SA113	Derivative of *S. aureus* NCTC 8325	Iordanescu and Surdeanu, 1976
SA113 Δ*atl*	*atlA* deletion mutant of strain SA113 (Δ*atlA*::spc)	Biswas et al., 2006
SA113 Δ*ypfP*	*ypfP* deletion mutant of strain SA113 *(ΔypfP::ermB)*	Fedtke et al., 2007
SEJ1	Protein A deficient derivative of *S. aureus* RN4220, markerless deletion of *spa*	Grundling and Schneewind, 2007a
SEJ1 Δ*ltaS* (4S5)	SEJ1-derived LTA-deficient suppressor strain	Corrigan et al., 2011
Staphylococcus simulans		
22	Indicator strain	Sahl and Brandis, 1981
Staphylococcus carnosus		
TM300	Indicator strain	Schleifer and Fischer, 1983

Table 3 continued

Strain or plasmid	Relevant characteristic(s)	Reference/Source
Micrococcus luteus		
DSM 1790	Indicator strain	DSMZ
Gram-negative bacteria		
Escherichia coli		
ATCC 25992	Indicator strain	ATCC
BL21(DE3)	Expression host for cytoplasmic proteins; λDE3-lysogen, T7 RNA polymerase under control of *lac* promotor	Studier and Moffatt, 1986
BW25113	K-12 strain	Datsenko and Wanner, 2000
BW25113 Δ*waaG*	*waaG* deletion mutant of strain BW25113 (Δ*waaG*::kan), lacks the LPS outer core	Baba et al., 2006
BW25113 Δ*waaP*	*waaP* deletion mutant of strain BW25113 (Δ*waaP*::kan); lacks LPS core phosphate	Baba et al., 2006
BW25113 Δ*waaY*	*waaY* deletion mutant of strain BW25113 (Δ*waaY*::kan), lacks one phosphate group in the LPS core region	Baba et al., 2006
ML-35pYC	Δ*lacI*, constitutive β-galactosidase expression, plasmid-encoded (pBR-22) β-lactamase, Amp^R	Lehrer et al., 1988
Plasmids		
pAH-PepI	Xylose-inducible expression of *pepI-gfp* fusion gene, Tet^R	Hoffmann et al., 2004
pet21b-PBP2	*pbp2* fused to C-terminal His_6, Amp^R	Körner, 2006

The following strains tested for the antimicrobial activity of oyster defensins were provided by the Ifremer (French Research Institute for Exploration of the Sea, University of Montpellier, France): *Bacillus megaterium* CIP 6620, *Escherichia coli* SBS 363, *Vibrio aestuarianus* CIP 102971, *Vibrio anguillarum* ATCC 19264, *Vibrio nigripulchritudo* CIP 103195 and *Vibrio splendidus* CIP 107715.

Table 4 Culture media used in this thesis.

Medium/Agar	Source/Composition
Columbia agar with 5% sheep blood	Becton Dickinson GmbH
Brain Heart Infusion (BHI)	12.5 g brain infusion solids; 5 g beef heart infusion solids; 10 g proteose peptone; 2 g glucose; 5 g NaCl; 2.5 g Na_2PO_4; pH 7.4. 37 g of the dehydrated medium (Oxoid) were dissolved in 1 l distilled water.
Lysogeny Broth (LB)	10 g tryptone; 5 g yeast extract; 10 g NaCl; ad 1 l distilled water; pH 7.5; ± 14 g agar.
Mueller-Hinton II Agar	Becton Dickinson GmbH
Mueller-Hinton Broth	300 g beef infusion; 17.5 g casein hydrolysate; 1.5 g starch; pH 7.3. 21 g of the dehydrated medium (Oxoid) were dissolved in 1 l distilled water.
Tryptone Soya Broth (TSB)	17 g pancreatic digest of casein; 3 g pancreatic digest of soya bean; 5 g NaCl; 2.5 g K_2HPO_4; 2.5 g glucose; pH 7.3. 30 g of the dehydrated medium (Oxoid) were dissolved in 1 l distilled water.

2.3.2 Sterilisation of media, equipment and bacterial cultures

Culture media, solutions, plastic vials and pipette tips were sterilised by autoclaving at 121°C for 20 min (Varioklav® 75S; H+P Labortechnik AG, Oberschleißheim, Germany). Glassware was incubated in a sterilisator (Kelvitron® t; Heraeus, Langenselbold, Germany) for 4 h at 180°C. Bacterial cultures and contaminated labware were autoclaved at 134°C for 30 min.

2.3.3 Determination of the optical density of a bacterial culture

The cell number of a bacterial culture was determined by measuring the optical density (OD) at a wavelength of 600 nm in a spectrophotometer (UV-160; Shimadzu, Duisburg, Germany). For staphylococci an OD_{600} of 1 corresponds to 1-2x 10^9 cells/ml (Brotz, 1997), and for *E. coli* an OD_{600} of 1 corresponds to 5x 10^8 cells/ml (personal communication A. Tossi, University of Trieste).

2.3.4 Determination of the minimal inhibitory concentration

The minimal inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial substance that will inhibit the visible growth of a microorganism.

MIC determinations were carried out in 96-well polypropylene microtiter plates (Nunc™; Thermo Fisher Scientific, Schwerte, Germany) by standard broth microdilution using half-

concentrated Mueller-Hinton broth or 10% Mueller-Hinton broth (diluted in 10 mM sodium phosphate buffer (SPB), pH 7.4). Test strains were grown to an OD_{600} of 1 and subsequently diluted to 1-2x 10^5 cells/ml. Then, 50 µl of the bacterial suspension were mixed with 50 µl of the peptide solution, and the inoculated microtiter plate was incubated for 10 min at RT on a microtiter shaker (Titertek; Flow Laboratories, Meckenheim, Germany). The MIC was read after 24 h of incubation at 37°C without agitation. The results given are mean values of at least two independent experiments performed in duplicate.

2.3.5 Antagonisation of putative target molecules

Different peptidoglycan precursors (C_{55}-P, lipid II, UDP-MurNAc-pp and UDP-GlcNAc) as well as LTA from *S. aureus* were tested for antagonisation of defensin antimicrobial activity. Therefore, serial dilutions of defensins were performed from 0.25 to 8x MIC in a polypropylene microtiter plate (Nunc™; Thermo Fisher Scientific, Schwerte, Germany) with the potential antagonist in a 1:1, 1:2 or 1:4 molar ratio. *S. aureus* SG511-Berlin was then added to the microtiter plate as for a conventional MIC determination. After a 24 h-incubation at 37°C, the lowest peptide/antagonist molar ratio that inhibited the antimicrobial activity of the highest defensin concentration (8x MIC) was determined.

2.3.6 Bacterial killing kinetics

S. aureus SG511-Berlin was grown in half-concentrated Mueller-Hinton broth to an OD_{600} of 0.1. Defensins were added in concentrations corresponding to 5x or 10x MIC (as determined after 24 h). At defined time intervals, 40 µl aliquots of the culture were taken, diluted in 360 µl 10 mM potassium phosphate buffer (pH 7) and 100 µl of appropriate dilutions were plated in triplicate on Mueller-Hinton II agar plates (Becton Dickinson GmbH). The plates were incubated overnight at 37°C and the number of colony forming units (CFU) was calculated based on the respective dilution factor. An untreated culture was used as a control.

2.3.7 Growth kinetic measurement

Cells were grown in half-concentrated Mueller-Hinton broth to the exponential phase and then diluted to an OD_{600} of 0.2 in 10% Mueller-Hinton broth (in 10 mM SPB, pH 7.4). Afterwards, 100 µl of the cell suspension were added to 100 µl of various antimicrobial compounds at 5x MIC. OD_{600} measurements were performed on a microplate reader (Sunrise™; Tecan, Crailsheim, Germany) over a period of 6 h at 37°C. Obtained data were analysed by Magellan™ data analysis software (Tecan, Crailsheim, Germany).

For determination of IC_{50} values, a serial dilution of hBD3 (0-2 µM) was performed in 10% Mueller-Hinton broth (in 10 mM SPB, pH 7.4). Then, cells were added to a final concentration of 1x 10^6 cells/ml and the bacterial suspension was incubated on a microplate reader as described above. The IC_{50} value was defined as the concentration which inhibited 50% of bacterial growth.

2.3.8 Potassium release from whole cells

Potassium efflux of whole cells was monitored with a MI-442 potassium electrode and a MI-409F reference electrode (Microelectrodes Inc., Bedford, USA) connected to a microprocessor pH meter (pH 213; HANNA® Instruments, Kehl am Rhein, Germany). Before starting the measurement, the electrodes were pre-conditioned in choline buffer (300 mM choline chloride, 30 mM MES, 20 mM Tris; pH 6.5) for at least 1 h. Calibration of the electrodes was carried out before each determination using freshly prepared standard solutions containing 0.01, 0.1 and 1 mM KCl in choline buffer (see above).
S. simulans 22 was grown in 50 ml half-concentrated Mueller-Hinton broth (± 10 mM glucose) at 37°C to an OD_{600} of 1 to 1.5. Then, cells were harvested by centrifugation (4,000 rpm, 3 min, 4°C), washed with 25 ml prechilled choline buffer and resuspended in choline buffer (± 10 mM glucose) to a final OD_{600} of 30. For each measurement, cells were diluted in choline buffer (± 10 mM glucose) to an OD_{600} of 3, and the potassium release was monitored for 5 min at RT. Defensins were added at 5x and 10x MIC. Potassium concentrations were calculated from the measured voltage according to Orlov et al. (2002) and expressed relative to the total amount of potassium released after addition of 1 µM of the pore-forming lantibiotic nisin (100% efflux).

2.3.9 Determination of the membrane potential using tetraphenyl-phosphonium bromide

S. aureus SG511-Berlin was grown in half-concentrated Mueller-Hinton broth to an OD_{600} of 0.5 to 0.6. To monitor the membrane potential, 1 µCi/ml of [^{3}H]tetraphenylphosphonium bromide (TPP^+; 26 Ci/mMol; Hartmann Analytic) was added (the lipophilic TPP^+ diffuses across the bacterial membrane in response to a trans-negative membrane potential). The culture was treated with defensins at 10x MIC, sample aliquots of 100 µl were filtered through cellulose acetate filters (pore size 0.2 µm; Whatman™, Dassel, Germany) and washed twice with 5 ml of 50 mM potassium phosphate buffer (pH 7). The filters were dried, placed into 5 ml scintillation fluid (Filtersafe, Zinsser Analytic) and the radioactivity was measured with a liquid scintillation counter (Tri-Carb 1900CA; Packard, Downers Grove, USA) for 5 min per filter. Non specific TPP^+ binding was determined by measuring

the TPP^+ incorporation into cells treated with 10% butanol (v/v); the total radioactivity was measured using unfiltered 100 µl sample aliquots. The pore-forming lantibiotic nisin (40 µg/ml corresponds to 10x MIC) was used as a control. For calculation of the membrane potential ($\Delta\psi$), the TPP^+ concentrations were applied into the Nernst equation $\Delta\psi = (2.3 \times R \times T/F) \times \log (TPP^+ \text{ inside}/TPP^+ \text{ outside})$, where *T* is the absolute temperature, *R* is the universal gas constant and *F* is the Faraday constant (according to Sahl, 1985).

2.3.10 Incorporation of radio-labelled metabolites

The effect of defensins on macromolecular synthesis was studied by monitoring the incorporation of [^{3}H]- or [^{14}C]-labelled precursors (2-[^{14}C]thymidine, 5-[^{3}H]uridine, L-[^{14}C]isoleucine; Hartmann Analytic) into *S. aureus* cells. Therefore, cultures of *S. aureus* SG511-Berlin were grown in half-concentrated Mueller-Hinton broth to an OD_{600} of 0.4, diluted 10-fold into fresh medium and allowed to regrow to an OD_{600} of 0.1. Subsequently, the respective labelled precursor was added to give a final concentration of 1 µCi/ml for ^{3}H-labelled metabolites and 0.1 µCi/ml for ^{14}C-labelled metabolites. Cultures were then split; one culture was treated with defensins, another one was run as a control. Selectivity of incorporation was confirmed using antibiotics that inhibit specifially protein (tetracycline), RNA (rifampicin) or DNA synthesis (ciprofloxacin).
Incorporation of the metabolites was monitored for up to 2 h. At certain time points, aliquots of 200 µl were taken and immediately added to 2 ml ice-cold 10% trichloroacetic acid. After 30 min incubation on ice, the samples were filtered through glass microfibre filters (Whatman™, Dassel, Germany) and washed with 5 ml 2.5% trichloroacetic acid. The dried filters were placed into 5 ml scintillation fluid (Filtersafe; Zinsser Analytic) and counts were obtained in a liquid scintillation counter (Tri-Carb 1900CA; Packard, Downers Grove, USA) for 5 min for each filter.

2.3.11 Membrane permeabilisation assay

The effect of defensins on the cell integrity of Gram-negative bacteria was determined photometrically by measuring the hydrolysis of the extracellular substrates CENTA™ (Calbiochem®) and o-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma-Aldrich) in *E. coli* ML35-pYC. The lactose permease-deficient strain contains a cytoplasmic β-galactosidase which accepts the lactose-mimic ONPG as substrate. ONPG is unable to cross the membrane and can only be hydrolysed in cells with damaged outer and inner membrane. Moreover, the strain harbours a plasmid-encoded periplasmic β-lactamase that hydrolyses the chromogenic cephalosporin CENTA™ if the outer membrane is impaired.

E. coli ML35-pYC was grown to early exponential phase and diluted to 1x 10^7 cells/ml in 10% Mueller-Hinton broth (in 10 mM SPB, pH 7.4). Afterwards, 20 µl of the cell suspension were incubated with either 0.15 mM CENTA or 1.5 mM ONPG and different defensin concentrations in a final volume of 200 µl. Hydrolysis was measured at 405 nm at 37°C for 120 min on a microplate reader (Sunrise™; Tecan, Crailsheim, Germany). Obtained data were analysed by Magellan™ data analysis software (Tecan, Crailsheim, Germany).

2.3.12 Intracellular accumulation of the final soluble cell wall precursor UDP-N-acetylmuramyl-pentapeptide

UDP-N-acetylmuramyl-pentapeptide (UDP-MurNAc-pp) is the final soluble precursor of bacterial cell wall biosynthesis. Antibiotics such as vancomycin, that interfere with the late, membrane-bound steps of peptidoglycan synthesis, trigger the accumulation of this precursor in the cytoplasm which can be isolated and detected by HPLC (Kohlrausch and Holtje, 1991).

For analysis of the cytoplasmic peptidoglycan precursor pool, *S. aureus* SG511-Berlin or *S. simulans* 22 was grown in half-concentrated Mueller-Hinton broth to an OD_{600} of 0.5 and supplemented with 130 µg/ml of chloramphenicol. Chloramphenicol prevents the *de novo* synthesis of enzymes that may interfere, e.g. through induction of cellular autolysis, with the accumulation of the UDP-linked peptidoglycan precursor in the cytoplasm (Dai and Ishiguro, 1988). After 15 min of incubation, defensins or vancomycin, respectively, were added at 10x MIC (to a final volume of of 5 ml) and the samples were further incubated for 30 min. Then, cells were spun down (5,300 rpm, 15 min, 4°C), resuspended in 0.25 ml MilliQ water and treated with two volumes of boiling water for 15 min.

The suspensions were cooled down and the cell extracts were adjusted to pH 2 by addition of H_3PO_4. Insoluble components were removed by centrifugation at 13,000 rpm for 5 min. The supernatants were filtered (Arodisc® syringe filter, pore size 0.2 µm; Pall, Dreieich, Germany) and analysed by reversed-phase high pressure liquid chromatography (RP-HPLC) in 50 mM sodium phosphate buffer pH 5.2, developed in an isocratic mode over 35 min at a flow rate 1 ml/min on a Nucleosil 100-C18 column (Schambeck SFD GmbH, Bad Honnef, Germany). UDP-linked cell wall precursors were detected at 260 nm and corresponding fractions were confirmed using matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS; Biflex™, Bruker Daltonics, Bremen, Germany) in a negative mode with 6-aza-2-thiothymine dissolved in 50% ethanol/20 mM ammonium citrate (v/v) as matrix.

2.4 Methods in molecular genetics

2.4.1 Determination of concentration and purity of nucleic acids

The concentration of nucleic acids was determined photometrically at 260 nm using a NanoDrop® ND-1000 spectrophotometer (Peqlab Biotechnologie, Erlangen, Germany). Purity was assessed by the ratio of absorbance at 260 nm and 280 nm (A_{260}/A_{280}).

2.4.2 Agarose gel electrophoresis

DNA fragments were analysed by standard agarose gel electrophoresis employing 0.8-2% agarose gels (w/v, Top Vision™ Agarose; Fermentas). Gels were run in 1x TAE buffer and the DNA was stained by incubating the gel 20-30 min in an ethidium bromide solution (1 µg/ml in 1 l MilliQ water). Subsequently, DNA bands were visualised using an ImageMaster® VDS (GE Healthcare/Pharmacia, Freiburg, Germany).

2.4.3 Isolation of plasmid DNA

2 to 5 ml of a bacterial overnight culture were harvested by centrifugation (13,000 rpm, 5 min, RT) and plasmids were extracted using GeneJET™ Plasmid Miniprep Kit (Fermentas) according to the manufacturer´s instructions. Finally, plasmid DNA was eluted with 30 µl MilliQ water (70°C) and stored at -20°C until further use.

2.4.4 Preparation of electrocompetent *E. coli* cells

For preparation of electrocompetent cells, *E. coli* BL21(DE3) was grown in 500 ml LB at 37°C to an OD_{600} of 0.5. Afterwards, the culture was incubated on ice for 15 min and subsequently harvested by centrifugation (6,000 rpm, 15 min, 4°C). Bacterial pellets were washed with 250 ml prechilled distilled water and 10 ml prechilled 10% glycerol (v/v). Finally, cells were resuspended in 800 µl prechilled 10% glycerol (v/v). Aliquots of 50 µl were frozen in liquid nitrogen and stored at -70°C until further use.

2.4.5 Transformation of *E. coli* by electroporation

Electrocompetent cells were thawed on ice and mixed with 1-2 µl plasmid DNA. After 1 min incubation on ice, the cells were electroporated in a MicroPulser™ (program Ec2; Biorad, München, Germany) using a prechilled electroporation cuvette (2 mm gap). Immediately, cells were removed from the cuvette and resuspended in 1 ml LB to recover for 1 h at 37°C on a rotary shaker.

Finally, 100 µl of the diluted (1:10, 1:100; v/v) and undiluted bacterial solution were streaked out on LB agar plates containing the appropriate antibiotic.

2.5 Protein and biochemical methods

2.5.1 Heterologous expression and purification of His-tagged PBP2

E. coli BL21(DE3) containing pet21b-PBP2 was grown in 1 l LB supplemented with 40 µg/ml ampicillin at 37°C to an OD_{600} of 0.6. Protein expression was induced by addition of IPTG (Fermentas) in a final concentration of 1 mM. After 4 h incubation, cells were pelleted (7,000 rpm, 12 min, 4°C; Sorvall Evolution RC; Heraeus, Langenselbold, Germany) and resuspended in 20 ml lysis buffer (Table 5). Then, 200 µg/ml lysozyme, 10 µg/ml RNase and 100 µg/ml DNase were added and cells were further incubated for 30 min at 37°C. After sonication (8x 10 s intervals at 60% including 15 s of cooling on ice; Sonifier® W250; G. Heinemann, Schwäbisch Gmünd, Germany), the cell debris was removed by centrifugation (10,300 rpm, 20 min, 4°C) and the supernatant was applied to 1.5 ml Ni-NTA-agarose (Qiagen) and incubated overnight at 4°C under stirring. The batch was transferred to a polypropylene column (Qiagen, Hilden, Germany) and washed with 10 ml lysis buffer and 5 ml washing buffer (Table 5) to remove weakly bound material. Recombinant proteins were eluted with 3 ml elution buffer (Table 5) in six fractions of 500 µl, mixed 1:1 with sterile glycerol (v/v) and stored at -20°C. Protein-containing fractions were pooled and dialysed using Slide-A-Lyzer dialysis cassettes (Pierce; Thermo Fisher Scientific, Schwerte, Germany).

Table 5 Buffers used for protein purification.

Buffer	Chemical ingredients
Lysis buffer	50 mM Tris-HCl (pH 7.5); 0.5 M NaCl; 1% Triton X-100 (v/v); 10 mM imidazole
Washing buffer	50 mM Tris-HCl (pH 7.5); 0.5 M NaCl; 1% Triton X-100 (v/v); 20 mM imidazole
Elution buffer	50 mM Tris-HCl (pH 7.5); 0.5 M NaCl; 1% Triton X-100 (v/v); 200 mM imidazole
Dialysis buffer	50 mM Tris-HCl (pH 7.5); 0.5 M NaCl; 1% Triton X-100 (v/v).

2.5.2 Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis

Purified proteins were analysed by discontinuous sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Table 6). Therefore, proteins were mixed with 4x

NuPAGE® LDS sample buffer (Invitrogen) and incubated for 5 min at 95°C prior to loading. The gel was run in Tris-glycine buffer (25 mM Tris; 192 mM glycine; 0.1% SDS (w/v); pH 8.5) in a Mini-Protean II Electrophoresis Cell (Bio-Rad, München, Germany) at 100 V for the stacking gel and 120 V for the resolving gel. Protein bands were visualised using PageBlue™ protein staining solution (Fermentas) according to the manufacturer´s instructions.

Table 6 Chemical ingredients for one resolving and one stacking gel.

Chemicals	Resolving gel (12%)	Stacking gel (4%)
Distilled water	3.845 ml	1.865 ml
3 M Tris, pH 8.5	1.25 ml	-
0.1 M Tris, 0.8% SDS (w/v)	-	0.3 ml
20% SDS (w/v)	0.05 ml	-
APS (21 mg/ml)	0.2 ml	0.08 ml
TEMED	0.005 ml	0.005 ml
40% acrylamide/bisacrylamide (w/v)	2.25 ml	0.25 ml

2.5.3 Zymogram analysis

Cell wall lytic enzymes in the supernatant of RTD- and Pep5-treated cells were analysed by zymograms. For this, *S. aureus* SG511-Berlin was grown in half-concentrated Mueller-Hinton broth to an OD_{600} of 0.6. Then, cells were harvested by centrifugation (4,000 rpm, 5 min, 4°C) and washed three times with 10 mM SPB (pH 7.4). Finally, cells were resuspended in 10% Mueller-Hinton broth (in 10 mM SPB, pH 7.4) and aliquots of 2.5 ml were incubated with peptides corresponding to 10x MIC. After incubation for 30 or 60 min, cells were pelleted (10,000 rpm, 5 min, 4°C). The supernatant containing released proteins was concentrated to a volume of 50 µl using VivaSpin-columns (MWCO 3,000; Sartorius, Göttingen, Germany) according to the manufacturer´s instructions. Subsequently, 25 µl of the enzyme extract were loaded onto a 12% polyacrylamide gel containing heat-killed *M. luteus* DSM 1790 cells as substrate. After run, the gel was washed three times with distilled water for 15 min before an overnight incubation in buffer (50 mM Tris-HCl, pH 7.5; 10 mM $CaCl_2$, 10 mM $MgCl_2$, 0.1% Triton X-100, v/v) at 37°C. Lytic activity was observed as clear zones against an opaque background. To gain a higher contrast, gels were stained with 0.1% methylene blue (w/v) for 15 min and washed with distilled water until clear bands became visible.

2.5.4 Determination of peptide and protein concentration

2.5.4.1 Bradford assay

The total protein concentration was measured using protein assay dye reagent (Bio-Rad, München, Germany) as recommended by the manufacturer. The assay is based on the absorbance shift of Coomassie Brilliant Blue G-250 bound to basic and aromatic amino acid residues. The absorption at 595 nm was measured with a spectrophotometer (UV-160; Shimadzu, Duisburg, Germany). A standard curve was obtained using different dilutions (0.125-2 mg/ml) of bovine serum albumin (BSA; Fermentas).

2.5.4.2 BCA protein assay

As detergents and some buffers interfere with the Bradford assay, protein concentrations were alternatively determined using the BCA™ Protein Assay Kit (Pierce) according to the manufacturer´s instructions. This quantification is based on the reduction of copper (II) ions that form a violet-coloured complex in alkaline solution. The absorption was measured with a NanoDrop® ND-1000 spectrophotometer (program: protein BCA; Peqlab Biotechnologie, Erlangen, Germany) at 562 nm. A standard curve was obtained using different concentrations (5-250 µg/ml) of BSA.

2.5.5 Peptide quantification by spectrometry

The spectrophotometer NanoDrop® ND-1000 (program: protein A280; Peqlab Biotechnologie, Erlangen, Germany) was used to measure peptide concentrations based on the molecular weight and extinction coefficient of the sample.

2.5.6 *In vitro* lipid II synthesis and purification

The cell wall precursor lipid II was synthesised *in vitro* using membrane preparations of *M*. *luteus* DSM 1790 containing the enzymes MraY and MurG and by external addition of the lipid carrier C_{55}-P.

2.5.6.1 Membrane preparation of *Micrococcus luteus* DSM 1790

For membrane preparation, 2 l TSB were inoculated with *M. luteus* DSM 1790 and incubated overnight at 37°C in a water bath (120 rpm). The following day, cells were harvested by centrifugation (8,000 rpm, 10 min, 4°C; Sorval Evolution RC; Heraeus, Langenselbold, Germany) and washed with 300 ml buffer (50 mM Tris-HCl; 10 mM $MgCl_2$; pH 7.5). Then, cells were resuspended in 100 ml buffer containing 400 µg/ml lysozyme

and 10 µg/ml DNase, and incubated on ice for 1 to 2 h. The cell suspension was subsequently warmed up to 35°C, cooled on ice again and membranes were centrifuged (18,000 rpm, 20 min, 4°C; Sorvall Evolution RC; Heraeus, Langenselbold, Germany). Finally, the pellets were resuspended in 8 ml buffer (see above) and stored at -70°C until further use.

2.5.6.2 Extraction of UDP-MurNAc-pp

S. simulans 22 was grown in 3 l Mueller-Hinton broth to an OD_{600} of 0.75 and supplemented with 130 µg/ml chloramphenicol. After 15 min incubation, vancomycin was added in a final concentration of 5 µg/ml and the cells were further incubated for 60 min. Afterwards, the cells were harvested by centrifugation (8,000 rpm, 10 min, 4°C; Sorvall Evolution RC; Heraeus, Langenselbold, Germany) and the pellet was resuspended in 20 ml MilliQ water. The cell wall precursor UDP-MurNAc-pp was extracted with two volumes of boiling water. Finally, the suspension was cooled down, cell debris was removed by centrifugation (18,000 rpm, 30 min, 4°C; Sorvall Evolution RC; Heraeus, Langenselbold, Germany) and the supernatant was lyophilised (Alpha 2-4 LSC; Christ, Osterode am Harz, Germany). The lyophilisate was dissolved in 4 ml MilliQ water and added to the *in vitro* lipid II synthesis (section 2.5.6.3).

2.5.6.3 *In vitro* lipid II synthesis

Initially, the lipid II synthesis was performed in an analytical scale (in a total volume of 75 µl) to find the optimal conditions as membrane preparations can vary in their synthesis rate (Table 7). Therefore, C_{55}-P (dissolved in methanol:chloroform, 1:1, v/v) was vacuum-dried and redissolved by addition of Triton X-100 (Table 7). After addition of the isolated membranes, the other ingredients were added in the following order: Tris-HCl, UDP-GlcNAc, $MgCl_2$ and UDP-MurNAc-pp (Table 7). The reaction mixture was subsequently incubated for 2 h at 30°C in a water bath. Bactoprenol-containing products were extracted with the same volume n-butanol/pyridine acetate (2:1, v/v; pH 4.2) and analysed by thin-layer chromatography (TLC). For the extraction solution 6 M glacial acetic acid was adjusted to pH 4.2 by addition of pyridine and mixed with two volumes of n-butanol.
After centrifugation (13,000 rpm, 3 min), 20 µl of the upper (butanol) phase were spotted on a silica gel (TLC Silica Gel 60 F_{254}; Merck, Darmstadt, Germany) and separated using chloroform:methanol:water:concentrated ammonium hydroxide (88:48:10:1, v/v/v/v) as the solvent (Rick et al., 1998). Lipid spots were visualised by PMA staining reagent (2.5% phosphomolybdate (w/v), 1% ceric sulfate (w/v), 6% sulphuric acid (v/v)). For this, the TLC

plate was dipped into PMA solution, dried and developed by heating at 140°C until dark blue spots appeared.

For synthesis and purification of milligram quantities of lipid II, the analytical procedure was scaled up by a factor of 250. After incubation of the reaction mixture, the butanol phase containing lipid II was collected, subsequently washed (8,000 rpm, 5 min, 4°C) with an equal volume of cold MilliQ water (pH 4.2) and purified as described below (section 2.5.6.4).

Table 7 Reaction mixture of lipid II synthesis (analytical scale).

Ingredients	**Volume (µl)**
1 mM C_{55}-P	5
4% Triton X-100	10
UDP-MurNAc-pp	5-10
10 mM UDP-GlcNAc	7.5
1 M Tris-HCl, pH 8	5
1 M $MgCl_2$	0.4
Membrane preparation of *M. luteus* DSM 1790	5-20
MilliQ water	ad 75

2.5.6.4 Purification of lipid II by HPLC

Lipid II was applied to a Hi-Trap DEAE column (5 ml; Amersham Biosciences, Freiburg, Germany) equilibrated with buffer A (chloroform:methanol:water, 2:3:1, v/v/v) with a flow rate of 3 ml/min. After sample application, the flow rate was set to 5 ml/min and the column was washed with buffer A for 30 min. Lipid II was eluted in a linear ammonium biocarbonat gradient (0-25% buffer B; chloroform:methanol:NH_4HCO_3, 2:3:1, v/v/v). 5 ml-fractions were collected and analysed by TLC as described above (section 2.5.6.3). Fractions containing lipid II were combined and concentrated using a rotary vacuum evaporator (Rotavapor RE11; Büchi Labortechnik, Flawil, Switzerland). Finally, lipid II was lyophilised, dissolved in chloroform:methanol (1:1, v/v) and stored in glas vials at -20°C. The lipid II concentration was determined by the phosphate content (section 2.5.7).

2.5.7 Phosphate determination

Phosphate determination was performed according to the method of Rouser (1970). For this, lipid samples were transferred to phosphate-free glass tubes and dried at 140°C for 20 min. Afterwards, 0.3 ml $HClO_4$ (70%, v/v) were added, and the tubes were incubated in a block heater at 180°C for 1 to 3 h. After cooling to RT, released inorganic phosphate

was reduced by addition of 1 ml MilliQ water, 0.4 ml ammonium molybdate (1.25%, w/v) and 0.4 ml of freshly prepared ascorbic acid (5%, w/v), followed by incubation in boiling water for 5 min. The absorption of the formed, blue-coloured complex was read at 795 nm. Phosphate concentrations of the samples were calculated by using a standard curve of 0-90 nmol inorganic phosphate (stock solution: 1 mM KH_2PO_4).

2.5.8 Inhibition of PBP2-catalysed reaction *in vitro*

The inhibition of the enzymatic activity of PBP2 *in vitro* was determined by incubating 2 nmol lipid II with 7.5 µg PBP2-His_6 in 10 mM MES (pH 5.5) in a final volume of 50 µl. Peptides were added in molar ratios ranging from 0.5 to 2 with respect to lipid II. The reaction mixtures were incubated at 30°C for 2 h and lipids were extracted by addition of one volume n-butanol/pyridine acetate (2:1, v/v) as described above (section 2.5.6.3). 20 µl of the upper phase were spotted onto a TLC plate (TLC Silica Gel 60 F_{254}; Merck, Darmstadt, Germany) and run in chloroform:methanol:water:ammonium hydroxide (88:48:10:1, v/v/v/v). Lipid bands were visualised by PMA staining (compare section 2.5.6.3).

2.5.9 Complexation of lipid II

Lipid II (2 nmol) was vacuum-dried for 5 min at RT and *Cg*-Defh2 in aqueous solution was added in molar ratios ranging from 0.1 to 1 with respect to the amount of lipid II. Reaction mixtures were vortexed and incubated at 30°C for 30 min and then applied onto TLC plates (TLC Silica Gel 60 F_{254}; Merck, Germany, Darmstadt). Plates were developed in butanol:acetic acid:water:pyridine (15:3:12:10, v/v/v/v) and stained with PMA (compare section 2.5.6.3).

2.5.10 Transmission electron microscopy

Cells were grown in half-concentrated Mueller-Hinton broth to an OD_{600} of 0.6. Aliquots of 5 ml were exposed to defensin (at 10x MIC) for 30 min or 60 min at 37°C. Afterwards, the bacteria were harvested by centrifugation (5,000 rpm, 5 min, 4°C), resuspended in 0.1 M SPB (pH 7.4) containing 3% glutaraldehyde (v/v; Sigma-Aldrich) and fixed overnight at 4°C. After washing the cells three times for 10 min with 0.1 M SPB (pH 7.4), they were postfixed in 2% phosphate-buffered osmium tetroxide (v/v; Sigma-Aldrich) at 4°C for 2 h. Subsequently, the samples were dehydrated with increasing concentrations of ethanol beginning with 30%. The dehydrated cells were incubated three times in propylene oxide for 5 min, followed by a treatment with a 1:1 mixture of polypropylene oxide and epon (v/v) overnight at RT. Finally, cells were embedded in epon using the EMbed-813 Embedding

Kit (Science Services, München, Germany) and incubated for polymerisation at 60°C for 48 h. Thin sections (60 nm) were obtained with a ultramicrotome (Ultracut R; Leica Microsystems, Bensheim, Germany) using a diamond knife (2 mm, 45°; Science Services, München, Germany).

The sections were transferred to copper grids (G200H-Cu; 3.05 mm; Science Services, München, Germany) and contrasted with 3% uranyl acetate (Ultrostain 1H; Leica Microsystems) and 0.3% lead citrate (10 ml distilled water; 30 mg lead(II) citrate tribasic trihydrate; 0.1 ml 10 M NaOH).

The sections were examined with an EM900 electron microscope (Zeiss; Oberkochen, Germany) at 50 kV.

For film developing, the negatives (Electron image film SO-163; Kodak; Science Services, München, Germany) were first placed into developer (Kodak D-19; Science Services) for 10 min, and subsequently rinsed with distilled water. Afterwards, they were incubated in a fixative solution (Agefix; Science Services) for 10-15 min, followed by incubation in a water tank for 20-25 min.

2.5.11 Biomolecular interaction analysis by surface plasmon resonance

The interaction between defensins and lipid II was analysed with a Bicacore® system which allows analysis of biomolecular interactions in real time based on a surface plasmon resonance (SPR). For this, one interaction partner is attached to the surface of a sensor chip (ligand), whereas the other interaction partner (analyte) is passed in solution over the surface with the immobilised ligand. The interaction between ligand and analyte generates a response which is proportional to the change in mass. In a sensogram the response (given in response units, RU; 1 RU = 1 pg/mm^2) is plotted against time.

Binding studies between *Cg*-Defs and lipid II were carried out on a Biacore® 2000 instrument (GE Healthcare, Freiburg, Germany) at 25°C. Therefore, a L1 sensor chip (GE Healthcare, Freiburg, Germany) was coated with lipids prepared as follows. Two kinds of small unilamellar vesicles (SUV) were prepared by sonication from 100% pure DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine; Avanti polar lipids) and from a mixture composed of DOPC and lipid II in a 99.2:0.8 molar ratio. SUV were coated on flow cell 1 and 2, respectively. Prior to immobilisation, the chip surface was cleaned by injection of 20 mM CHAPS for 5 min at a flow rate of 10 µl/min, followed by a 1 min injection of 30% ethanol at 10 µl/min. SUV were injected at 2 µl/min. To remove loosely bound vesicles, the surface was washed with 10 mM NaOH for 1 min at 100 µl/min. A short injection of BSA (100 µg/ml) at 30 µl/min was used to cover all non-specific binding sites. The coating of the lipid layers gave a response in the range of 6,000 to 6,500 RU. For kinetics,

peptides were simultaneously injected at 250 nM on the two flow cells at a flow rate of 30 µl/min. Dissociation was monitored over 400 s in HBS running buffer (10 mM HEPES pH 7.4; 300 mM NaCl; GE Healthcare). New lipid layers were prepared for every injection to avoid the need for a regeneration step. The response of peptide binding on DOPC layers was taken as a negative control and subtracted from the response on lipid II-containing DOPC layers. To determine the binding ratio, RU levels were measured at the plateau, just after the end of injection for the three defensins, nisin and tachyplesin. Two separate experiments were performed for each peptide injected.

2.5.12 Chromatographic purification of lipoteichoic acid

The extraction and purification of lipoteichoic acid (LTA) of *S. aureus* SG511-Berlin was performed according to the protocol of Grundling and Schneewind (2007b) with some modifications.

Cells were grown in 3 l BHI overnight at 37°C in a water bath (120 rpm). The following day, the bacteria were harvested by centrifugation (7,000 rpm, 15 min, RT) and washed with 0.1 M sodium citrate (pH 4.7). For LTA extraction, cells were lysed in a Precellys® 24 homogeniser (6,500 rpm, 3x 30 s, 30 s break; Peqlab Biotechnologie, Erlangen Germany) using the Precellys® glas kit (0.1 mm; Peqlab Biotechnologie, Erlangen, Germany). Glas beads were pelleted by centrifugation (7,000 rpm, 5 min) and the supernatant containing bacterial debris was centrifuged at 13,000 g for 60 min (Sorvall Evolution RC; Heraeus, Langenselbold, Germany). Then, the pellets were washed with 40 ml 0.1 M sodium citrate buffer (pH 4.7), centrifuged as described above and finally resuspended in 20 ml sodium citrate buffer (pH 4.7). The suspension was mixed with an equal volume of 1-butanol and incubated at RT for 30 min under stirring. After centrifugation (13,000 g, 20 min, RT; Sorvall Evolution RC), the aqueous (lower) phase containing LTA was dialysed against 20 mM sodium citrate (pH 4.7) using Slide-A-Lyzer dialysis cassettes (2,000 MWCO; Thermo Fisher Scientific, Schwerte, Germany).

Hydrophobic chromatography was executed on an octyl-sepharose CL 4B column (diameter: 1.6 cm, radius: 0.8 cm, length: 24 cm) equilibrated with 0.1 M sodium citrate (pH 4.7) in 15% propanol (v/v). The LTA-containing lysate was adjusted to 15% 1-propanol (v/v) and subjected to the column with a flow rate of 0.3 ml/min. After sample application, the column was washed overnight with 0.1 M sodium citrate (pH 4.7) in 15% propanol at the same flow rate. LTA was eluted using a 300 ml gradient of 15-80% 1-propanol in 0.1 M sodium citrate (pH 4.7) with a flow rate of 0.3 ml/min. 5 ml fractions were collected and analysed by their phosphate content (section 2.5.7). All phosphate-containing fractions were combined and dialysed against 0.1 M sodium citrate (pH 4.7).

Afterwards, the LTA were concentrated to a volume of 5 ml using VivaSpin columns (15R, MWCO 2,000; Sartorius, Göttingen, Germany) and stored at -20°C until further use.

2.5.13 Determination of glucose concentration

The glucose concentration of the isolated LTA was determined by the anthrone method according to Herbert et al. (1971). For this, 0.2 ml of sample and standard solutions (20-100 µg glucose) were chilled on ice and subsequently mixed with 1 ml freshly prepared anthrone reagent (200 mg anthrone; 5 ml ethanol; 95 ml 75% H_2SO_4, v/v). Afterwards, the tubes were incubated in a block heater at 100°C for 10 min. After cooling, the absorbance was read at 625 nm.

2.5.14 CF-efflux from LTA-containing liposomes

Vesicles were made of pure DOPC or DOPC supplemented with 0.5 mol% LTA (referring to the total amount of phospholipids). For this, 4 µM DOPC (dissolved in chloroform:methanol, 1:1, v/v) were mixed with LTA in a round bottom flask and the solvent was evaporated under a nitrogen stream. Final traces of solvents were removed in a dessicator for further 2 h and the dried lipid films were stored at -20°C until further use. The lipids were resuspended in 600 µl buffer (50 mM MES-KOH; 100 mM K_2SO_4; pH 6 or 10 mM Tris-HCl; 0.85% NaCl; pH 7.2) containing 50 mM carboxyfluorescein (CF) and the flask was subsequently placed on a rotary evaporator (without a vacuum; Rotavapor RE11; Büchi Labortechnik, Flawil, Switzerland) spinning for 30-60 min in a water bath at 30°C. The obtained multilamellar vesicles were subjected to 6 to 8 cycles of freeze-thaw, and then additionally passed 19 times through a polycarbonate filter (0.4 µm; Whatman™, Dassel, Germany) in an extrusion apparatus (Mini-Extruder; Avanti Polar Lipids, Alabaster, USA). Liposomes were separated from unencapsulated CF by gel filtration using sephadex G-50 (Sigma-Aldrich). Therefore, 2 ml of sephadex G-50 (equilibrated in buffer) were placed into polypropylene columns (Qiagen, Hilden, Germany) and centrifuged at 750 g for 3 min. The liposomes were subsequently applied to the column and centrifuged as described above. This procedure was repeated until the flow-through containing the vesicles was almost colourless.

Then, the CF-loaded vesicles were diluted in 1.5 ml buffer (see above) at a final concentration of 25 µM phospholipid on a phosphorous base. The fluorescence intensity was measured at 520 nm (excitation at 492 nm) on a RF-5301 spectrophotometer (Shimadzu, Duisburg, Germany) for 3 min at RT. Peptides were added after 25 s at concentrations of 1 µM. To determine 100% marker release, 20 µl of 20% Triton X-100 (v/v) were added at the end of each measurement.

3 Results

3.1 Part 1: Insight into invertebrate defensin mode of action

Defensins have been found in different phylogenetic groups of invertebrates. Interestingly, they can be classified according to their activity spectrum: antibacterial *versus* antifungal (Bulet et al., 2004).

To investigate the mechanism of action of antibacterial invertebrate defensins, a comparative study with three defensin variants *(Cg*-Defs*)* produced by the Pacific oyster *Crassostrea gigas* was performed in collaboration with Delphine Destoumieux-Garzón (University of Montpellier, France). *Cg*-Defh1 and *Cg*-Defh2 were isolated from the hemocytes (Gonzalez et al., 2007a), whereas *Cg*-Defm was identified from the oyster mantle (Gueguen et al., 2006). The oyster defensins consist of 43 amino acid residues and have a net charge of +1 to +3 (Figure 6). *Cg*-Defh1 and *Cg*-Defh2 share 84% and 79% sequence identity with *Cg*-Defm.

A

Defensin	Sequence	MW (Da)	Net charge
Cg-Defh1	GFGCPRDQYKCNSHCQSIGCRAGYCDAVTLWLRCTCTDCNGKK	4,763	+1
Cg-Defh2	GFGCPGDQYECNRHCRSIGCRAGYCDAVTLWLRCTCTGCSGKK	4,677	+2
Cg-Defm	GFGCPGNQLKCNNHCKSISCRAGYCDAATLWLRCTCTDCNGKK	4,642	+3

B

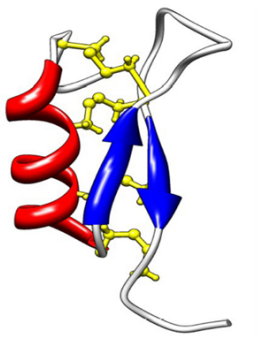

Figure 6 A: Amino acid sequence alignment, molecular weights and net charges (at pH 7) of three defensins produced by the oyster *C. gigas* (*Cg*-Defs). Residues with a positive charge are marked in red, negatively charged residues are marked in blue.
B: The 3D structure of *Cg*-Defm. The defensin adopts a cysteine-stabled α-helix β-sheet structure (CSαβ) in solution; the four disulphide bridges are marked in yellow (Schmitt et al., 2012b).

The oyster defensins adopt a typical CSαβ fold in solution also found in defensins from plants and fungi. In contrast to other invertebrate defensins, they possess four disulphide bonds (C1-C5, C2-C6, C3-C7, C4-C8) which probably confer a more stable structure under high-osmolarity sea water conditions (Figure 6).

3.1.1 Antibacterial activity spectrum of oyster defensins

The three oyster defensin variants, *Cg*-Defh1, *Cg*-Defh2 and *Cg*-Defm, were expressed as recombinant peptides in *E. coli* and then tested for their antimicrobial activity in a standard broth microdilution assay. All oyster defensins were active at low micromolar concentrations against the Gram-positive bacteria tested (Table 8), but did not show significant antimicrobial activity against Gram-negative bacteria, including the oyster pathogens *V. splendidus* and *V. aestuarianus* (Table 8). Interestingly, *Cg*-Defh2 was the most potent peptide, exhibiting 2- to 4-fold lower MIC values than *Cg*-Defh1 and *Cg*-Defm against the tested Gram-positive strains.

Table 8 Antimicrobial activity of the three oyster defensins *Cg*-Defh1, *Cg*-Defh2 and *Cg*-Defm. MIC values (µM) are expressed as the lowest concentration that caused visible growth inhibition.

	***Cg*-Defh1**	***Cg*-Defh2**	***Cg*-Defm**
Gram-positive bacteria			
B. megaterium CIP 6620	0.06	0.03	0.03
S. aureus SG511-Berlin	0.5	0.125	0.25
S. simulans 22	2	0.5	1
S. haemolyticus	6	2	2
Gram-negative bacteria			
E. coli SBS 363	40	20	20
V. aestuarianus CIP 102971	>40	>40	>40
V. anguillarum ATCC 19264	>40	>40	>40
V. nigripulchritudo CIP 103195	>40	>40	>40
V. splendidus CIP 107715	>40	>40	>40

3.1.2 Impact of oyster defensins on the membrane integrity of *S. aureus*

To test whether oyster defensins have deleterious effects on the membrane of Gram-positive bacteria, the membrane potential of *S. aureus* SG511-Berlin exposed to oyster defensins was determined. The membrane potential was monitored for a period of 20 min after peptide addition from the distribution of the lipophilic cation [^{3}H]tetraphenylphosphonium bromide (TPP^+) inside and outside the bacterial cells after

treatment. The pore-forming peptide nisin was used as a positive control. Oyster defensins did not cause any change in membrane potential when used at 10x MIC (Figure 7). In contrast, the addition of nisin at 10x MIC (40 µg/ml) induced a significant decrease of the membrane potential of *S. aureus* SG511-Berlin (Figure 7). Altogether, these data indicate that at lethal concentrations, oyster defensins do not compromise the membrane integrity of *S. aureus*.

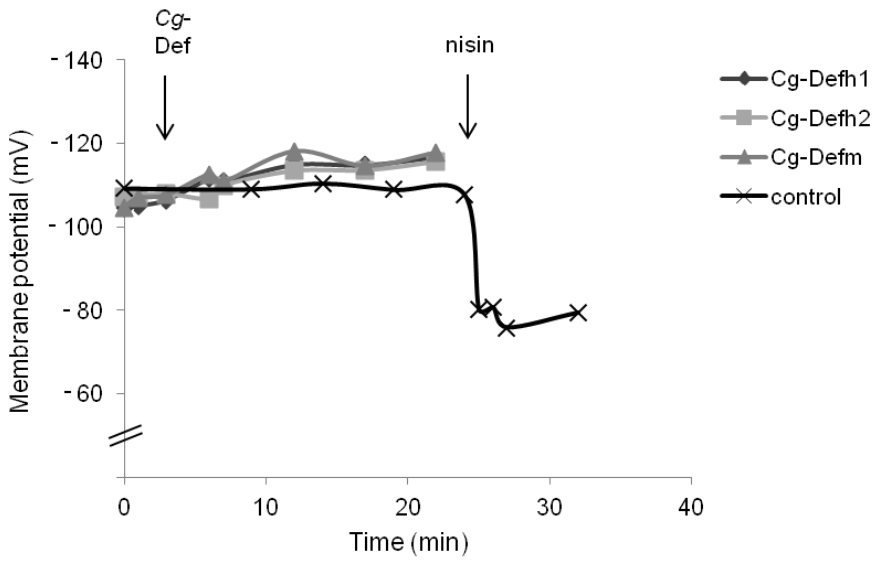

Figure 7 Influence of oyster defensins on the membrane potential of *S. aureus* SG511-Berlin. Cells were incubated with 10x MIC of *Cg*-Defs or the pore-forming lantibiotic nisin. The membrane potential was calculated from the distribution of the lipophilic cation [^{3}H]tetraphenylphosphonium bromide (TPP^+) inside and outside the cells. TPP^+ concentrations were inserted into to the Nernst equation. Arrows indicate the moment of peptide addition.

3.1.3 Accumulation of the soluble cell wall precursor UDP-N-acetylmuramyl-pentapeptide in oyster defensin treated cells

As the antibacterial activity of oyster defensins was mostly directed against Gram-positive bacteria (Table 8), the potential interference of the peptides with the peptidoglycan biosynthesis was investigated. The biosynthesis consists of a series of cytoplasmic reactions yielding soluble UDP-linked precursors of peptidoglycan, followed by membrane-bound steps, which starts with the anchoring of the last soluble precursor, UDP-N-acetylmuramyl-pentapeptide (UDP-MurNAc-pp), to the lipid carrier undecaprenyl phosphate (C_{55}-P).

To analyse the interference of oyster defensins with peptidoglycan biosynthesis, the accumulation of UDP-MurNAc-pp was monitored. Therefore, the cytoplasmic pool of soluble peptidoglycan precursors in cell extracts of defensin-treated *S. simulans* 22 was

analysed. RP-HPLC and mass spectrometry analysis of bacterial cell extracts showed that the three oyster defensins caused the intracellular accumulation of UDP-MurNAc-pp in a similar manner to vancomycin, which is known to form a complex with the cell wall precursor lipid II (Figure 8).

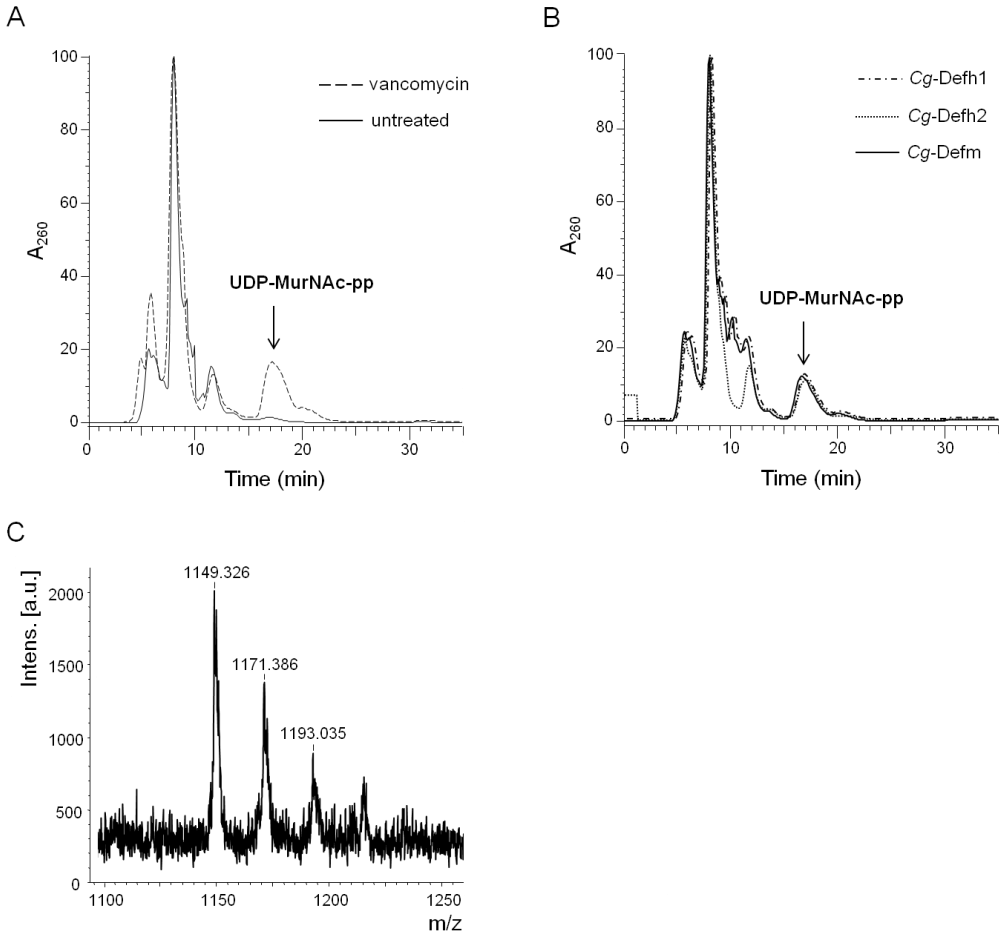

Figure 8 Intracellular accumulation of the ultimate soluble cell wall precursor UDP-MurNAc-pp in *S. simulans* 22 treated with oyster defensins. *S. simulans 22* was exposed to vancomycin (positive control; A) or *Cg*-Defs (B) at 10x MIC. Treated cells were extracted with boiling water and the cytoplasmic pool was analysed by RP-HPLC. Intracellular accumulation of UDP-MurNAc-pp (arrow) was confirmed by mass spectrometry (C; calculated monoisotopic mass 1149.35); in addition the mono- and disodium salts were detected.

The continuous biosynthesis and subsequent defensin-induced accumulation of UDP-MurNAc-pp in the cytoplasm is indicative of the absence of leakage and therefore consistent with the absence of membrane damage observed (Figure 7). Moreover, it strongly suggests that oyster defensins are inhibitiors of peptidoglycan biosynthesis. As the synthesis of UDP-MurNAc-pp itself is not impaired by oyster defensins, the cells cannot be de-energised and the inhibition may occur at the membrane-bound steps of the biosynthesis.

3.1.4 Antagonisation of antibacterial activity of oyster defensins

Antagonisation assays were performed to identify the putative targets of oyster defensins in peptidoglycan biosynthesis. Briefly, peptidoglycan synthesis occurs as follows: After the anchoring of UDP-MurNAc-pp to C_{55}-P, the resulting lipid I is converted into lipid II by addition of GlcNAc. Lipid II is subsequently translocated across the cytoplasmic membrane to the outer leaflet and incorporated into the growing peptidoglycan network by the activity of penicillin binding proteins (PBPs; Figure 27). Therefore, several intermediates of the peptidoglycan biosynthesis (namely lipid II, C_{55}-P, UDP-MurNAc-pp and UDP-GlcNAc) were used as potential antagonists in liquid growth inhibition assays. Lipid II was the only compound that prevented the oyster defensin antimicrobial activity (Table 9). Indeed, when used at 1:1 molar ratio, oyster defensins became unable to inhibit growth of *S. aureus* SG511-Berlin at concentrations as high as 8x MIC. Neither activated cell wall sugars (UDP-MurNAc-pp and UDP-GlcNAc) nor the lipid carrier C_{55}-P had similar antagonistic effects. This strongly suggests that the oyster defensins bind to the cell wall precursor lipid II.

Table 9 Antagonisation of the antimicrobial activity of oyster defensins against *S. aureus* SG511-Berlin by putative target molecules of cell wall biosynthesis. The following symbols are used: + for antagonisation; - for normal antimicrobial activity.

Antagonist	*Cg*-Defh1	*Cg*-Defh2	*Cg*-Defm	Molar ratio antagonist:*Cg*-Defs
C_{55}-P	-	-	-	-
lipid II	+	+	+	1:1
UDP-MurNAc-pp	-	-	-	-
UDP-GlcNAc	-	-	-	-

3.1.5 Binding of oyster defensins to lipid II

To further analyse the binding of oyster defensins to lipid II, the effect of *Cg*-Defs on the PBP2-catalysed reaction was determined *in vitro*. PBP2 is a bifunctional enzyme (transpeptidase and transglycosylase) that transforms monomeric lipid II into polymeric peptidoglycan. This reaction occurs at the outside of the cytoplasmic membrane and could likely be a target of oyster defensins. Therefore, purified recombinant PBP2-His_6 was incubated with lipid II and *Cg*-Defh2 in different molar ratios. After 2 h incubation, the lipids were extracted with n-butanol/pyridine acetate (2:1) and separated by TLC (compare section 2.5.8). However, in presence of *Cg*-Defh2 lipid II could not be extracted, suggesting the formation of a complex which is insoluble in aqueous solution (Figure 9).

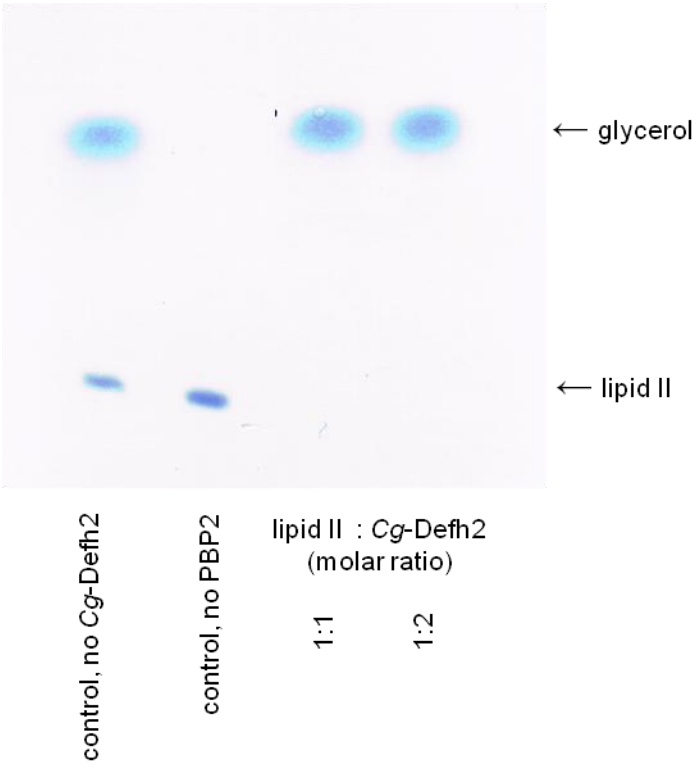

Figure 9 Inhibition of the PBP2-catalysed reaction by oyster defensins *in vitro*. *Cg*-Defh2 was incubated with lipid II and PBP2 in molar ratio of 1:1 and 1:2. In the presence of oyster defensin lipid II could not be extracted.

Thus, the binding of oyster defensins was assayed by incubating the oyster defensins with lipid II at molar ratios ranging from 1:0.1 to 1:2 and directly spotting on the TLC plate (without extraction). With the addition of increasing amounts of oyster defensins, the free lipid II band disappeared from the TLC plate and became undetectable at a 1:1 molar ratio as shown in Figure 10 for *Cg*-Defh2. These data indicate that lipid II and oyster defensins bind to each other in a 1:1 stoichiometry.

Moreover, surface plasmon resonance (SPR) experiments were performed to fully demonstrate the interaction on the binding of the three oyster defensin variants to lipid II and to obtain quantitative data of the binding process. Small unilamellar vesicles (SUV) of DOPC containing 0.8 mol% lipid II were prepared and immobilised on a SPR chip for interaction assays. The binding of oyster defensins was compared to that of nisin (positive control) and tachyplesin, an invertebrate LPS-binding antimicrobial peptide (negative control), at concentrations of 250 nM for each peptide. Although little to no binding of oyster defensins was observed on DOPC vesicles, a significant binding of the three defensin variants was observed on DOPC vesicles with 0.8 mol% lipid II.

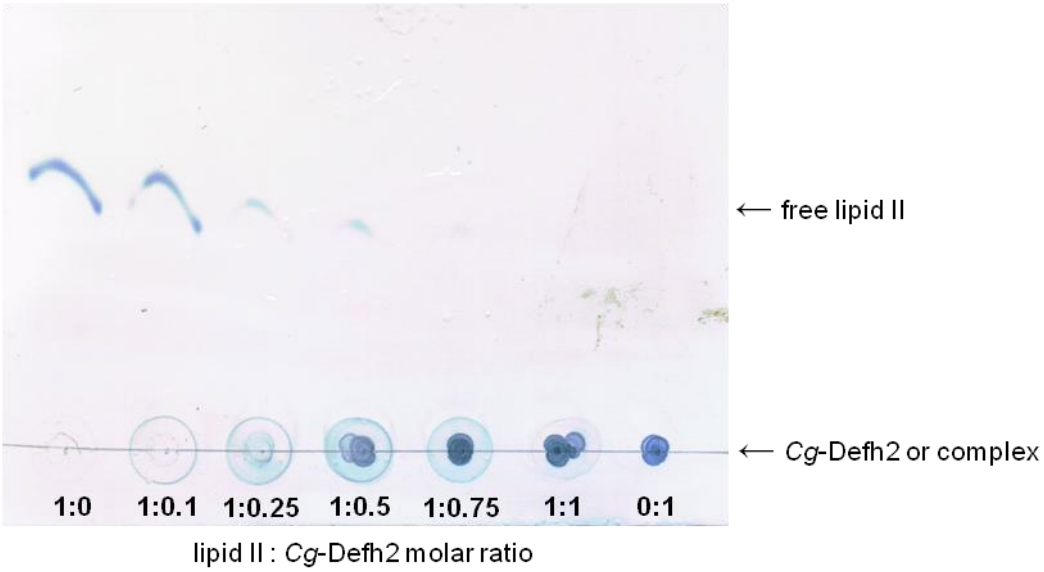

Figure 10 The oyster defensin-lipid II complex. Lipid II was incubated in the presence of *Cg*-Defh2 at molar ratios ranging from 1:0.1 to 1:1. The stable complex remained at the application spot, whereas free lipid II migrated up the TLC plate.

Overall, interaction kinetics were similar for defensins and nisin, although they differed by their association phase (Figure 11). The binding of oyster defensins appeared rather irreversible with no decay in the response units (RU) over time. As expected, tachyplesin did not bind to the DOPC vesicles containing 0.8 mol% lipid II, and even gave a negative signal most likely due to the membrane disruption properties of the peptide (Doherty et al., 2006) (Figure 11).

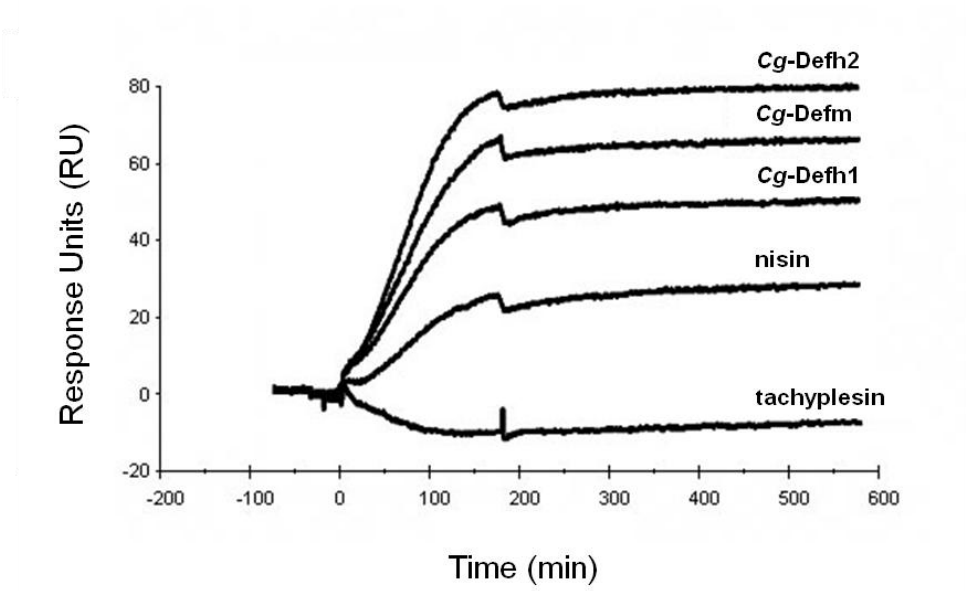

Figure 11 SPR analysis: Binding of oyster defensins to lipid II containing vesicles. The sensorgram depicts the interaction of oyster defensins, nisin (positive control) and tachyplesin (negative control) with immobilised DOPC vesicles containing 0.8 mol% lipid II. All peptides were added at 250 nM. The control sensorgrams (peptide interaction with immobilised DOPC vesicles) were subtracted from the data presented.

In two independent experiments, the three oyster defensins displayed higher binding to lipid II than nisin used as a positive control (2- to 4-fold higher RU). Interestingly, differential binding was also observed among the oyster defensin variants. Indeed, *Cg*-Defh2 and *Cg*-Defm, which were also the most active variants (Table 8), gave similar association levels to DOPC vesicles with 0.8 mol% lipid II (Figure 11). Altogether, this shows that oyster defensins are strong ligands of lipid II and that differential binding occurs between variants.

3.2 Part 2: Insight into the antistaphylococcal mode of action of θ-defensins

Theta-defensins are cyclic antimicrobial peptides composed of only 18 amino acids. They have been first isolated from the leukocytes and monocytes of rhesus macaques (RTDs, rhesus macque θ-defensin) (Tang et al., 1999; Tran et al., 2002) and subsequently found in many species of Old World monkeys (Garcia et al., 2008; Nguyen et al., 2003). They arose from a mutated α-defensin gene containing a premature stop codon in its defensin domain. During biogenesis a segment of 9 amino acids is excised from the defensin precursor and spliced head-to-tail to a similar or identical nonapeptide; the resulting homodimeric or heterodimeric products are stabilised by three disulphide bridges (Figure 12; Tran et al., 2002). Rhesus macaques express three θ-defensin precursors which can pair to generate six different peptides (RTD-1 to RTD-6). Among these defensins, RTD-1 is the most abundant (Tongaonkar et al., 2011). The peptides display a broad-spectrum activity against Gram-positive and Gram-negative bacteria, fungi and even enveloped viruses (Cole et al., 2002; Tang et al., 1999; Tran et al., 2002). Moreover, it has been demonstrated that the cyclic structure is crucial for their antimicrobial activity and confers salt resistance up to 150 mM NaCl (Tang et al., 1999).

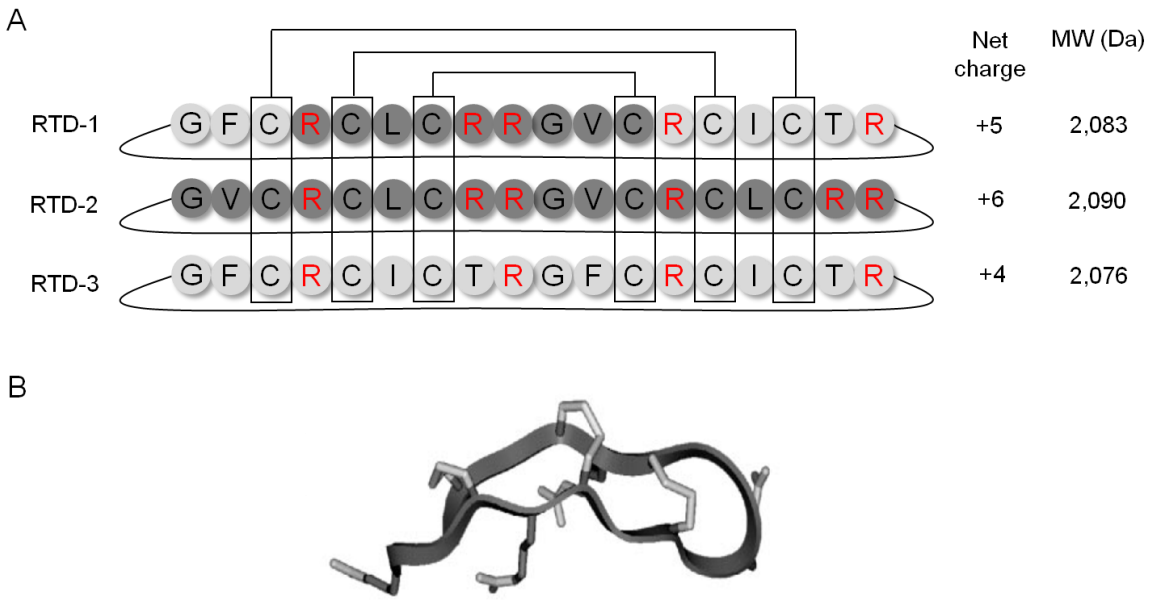

Figure 12 A: Amino acid sequence alignment, molecular weights and net charges (at pH 7) of heterodimeric RTD-1 and homodimeric RTD-2 and RTD-3; positively charged residues are marked in red. The peptide bond between G1 and R18 is indicated by lines (modified according to Tran et al., 2002).
B: 3D structure of RTD-1 (Cole et al., 2002).

In this study, the antistaphylococcal action of θ-defensins was investigated using the heterodimeric RTD-1 and homodimeric RTD-2 (Figure 12).

3.2.1 Antibacterial activity of θ-defensins against staphylococci

The two θ-defensin variants, RTD-1 and RTD-2, were initially tested for their activities against different staphylococcal species in a standard broth microdilution assay. Both peptides exhibited potent antimicrobial activity and inhibited growth of the three test strains at concentrations ranging from 0.5 to 6 µg/ml (corresponding to 0.24 to 2.88 µM; Table 10).

Table 10 Antimicrobial activity of RTDs against staphylococci in half-concentrated Mueller-Hinton broth. MIC values are expressed as the lowest concentration that caused visible growth inhibition.

Strain	**RTD-1**		**RTD-2**	
	µg/ml*	**µM**	**µg/ml***	**µM**
S. aureus SG511-Berlin	6	2.88	4	1.91
S. simulans 22	1.5	0.72	1.5	0.72
S. carnosus TM300	0.75	0.36	0.5	0.24

*Average values obtained from two or more independent experiments.

3.2.2 Impact of RTDs on staphylococcal cell wall biosynthesis

Recently, it has been shown that fungal (Schneider et al., 2010), invertebrate (section 3.1) and vertebrate defensins (de Leeuw et al., 2010; Sass et al., 2010) bind to and sequester the cell wall building block lipid II, thereby specifically inhibiting cell wall biosynthesis.
In order to prove whether θ-defensins also interfere with membrane-bound steps of peptidoglycan biosynthesis, the accumulation of the final soluble cell wall precursor UDP-MurNAc-pp was monitored in staphylococcal cells exposed to RTDs. *S. simulans* 22 and *S. aureus* SG511-Berlin were treated with defensins at 10x MIC and the cytoplasmic pool of UDP-linked peptidoglycan precursors was extracted and analysed by RP-HPLC (compare section 2.3.12). As it is shown in Figure 13, RTDs did not cause accumulation of UDP-MurNAc-pp in the cytoplasm compared to vancomycin-treated control cells. These data indicate that the antibiotic action of RTDs differ from that of the defensins mentioned above.

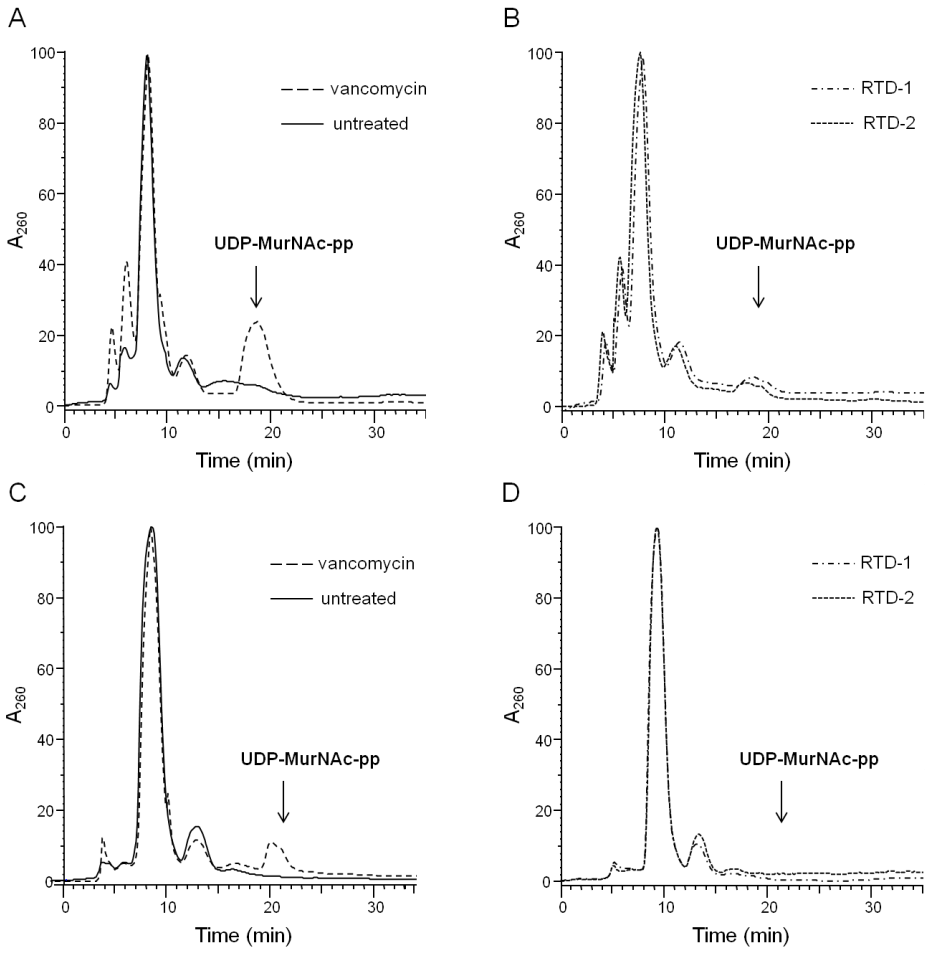

Figure 13 Intracellular accumulation of the final soluble cell wall precursor UDP-MurNAc-pp in *S. simulans* 22 (A, B) and *S. aureus* SG511-Berlin (C, D) exposed to θ-defensins. Cells were treated with 10x MIC vancomycin (positive control; A, C) or RTDs (B, D), incubated for 30 min, and subsequently extracted with boiling water. The cytoplasmic pool was analysed by RP-HPLC. Intracellular accumulation of UDP-MurNAc-pp (arrow) was confirmed by mass spectrometry (data not shown).

3.2.3 Impact of RTDs on macromolecular synthesis

To investigate the effect of RTDs on intracellular bacterial biosynthetic reactions, the incorporation of ^{14}C-thymidine, ^{3}H-uridine and ^{14}C-isoleucine into DNA, RNA and proteins of *S. aureus* SG511-Berlin was measured over a period of 2 h after peptide addition.

Interestingly, all three biosynthesis pathways were simultaneously affected in a concentration-dependent manner (data at 1x MIC are shown in Figure 14), suggesting that in presence of RTDs cells are either unable to actively take up biosynthetic precursors or that macromolecular synthesis is generally impaired. Thus, the θ-defensin activity is distinct from the action of antibiotics that interfere specifically with one of the pathways.

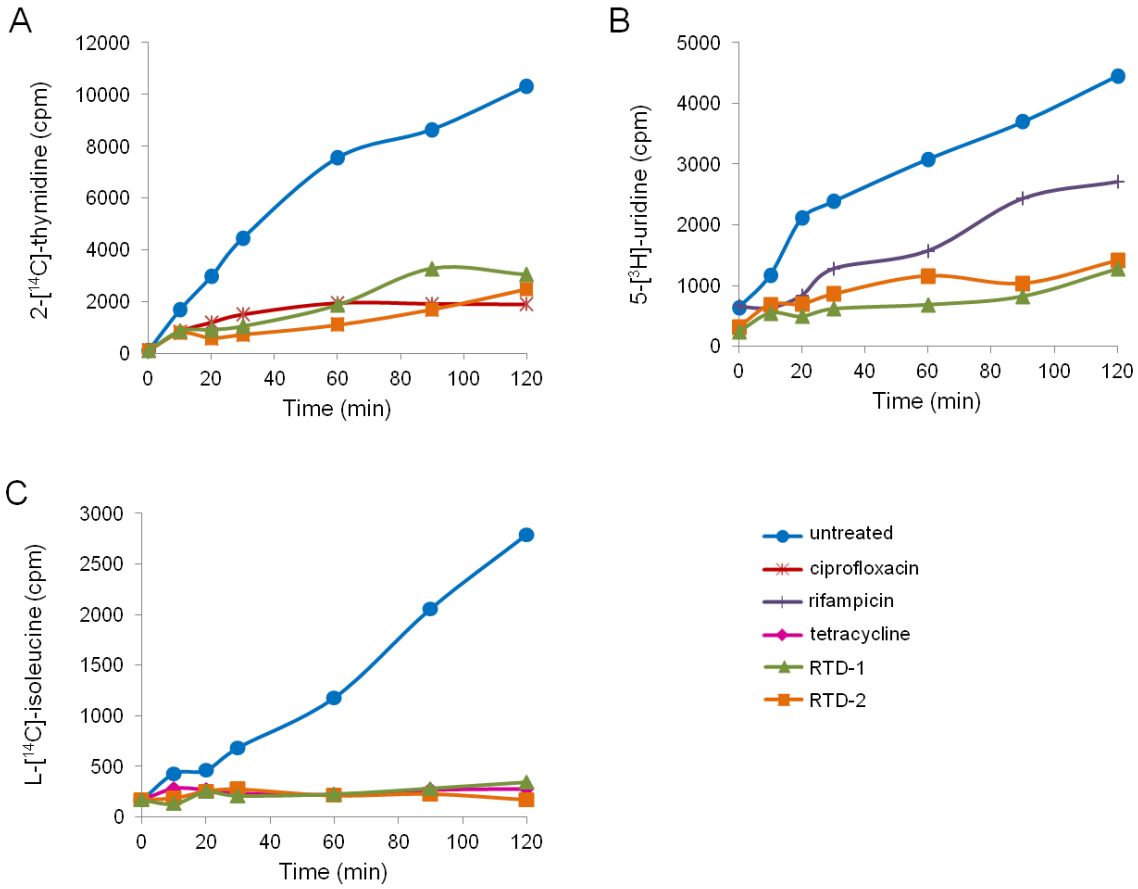

Figure 14 Influence of RTDs on the macromolecular synthesis in *S. aureus* SG511-Berlin. The incorporation of ^{14}C-thymidine into DNA (A), ^{3}H-uridine into RNA (B) and L-^{14}C-isoleucine into cellular proteins (C) of untreated and RTD-treated cells was measured over a period of 2 h. Antibiotics that inhibit particularly one of the pathways were used as controls.

3.2.4 Impact of RTDs on the membrane integrity

Effects on the membrane integrity impair energy-dependent transport processes. Hence, permeabilisation of the bacterial membrane would explain the rapid and simultaneous inhibition of all biosynthetic reactions in the presence of RTDs. To assess the membrane impairment by θ-defensins, the potassium release of whole cells was monitored over a period of 5 min by growing *S. simulans 22* in half-concentrated Mueller-Hinton broth and subsequently diluting the cells in choline buffer (compare section 2.3.9).

Under these conditions, significant potassium efflux could not be observed in response to RTDs at 5x and 10x MIC (Figure 15 A). However, energisation of the cells by the addition of 10 mM glucose resulted in rapid concentration-dependent ion release after peptide treatment (Figure 15 B). Notably, RTD-1 had a stronger impact on the membrane integrity of *S. simulans 22* than RTD-2. At concentrations corresponding to 10x MIC, RTD-1 induced almost 90% ion leakage while RTD-2 led to 70% of potassium release after 5 min

incubation. In contrast, the activity of the pore-forming lantibiotic nisin - used here as a positive control - was independent of the presence of glucose (Figure 15).

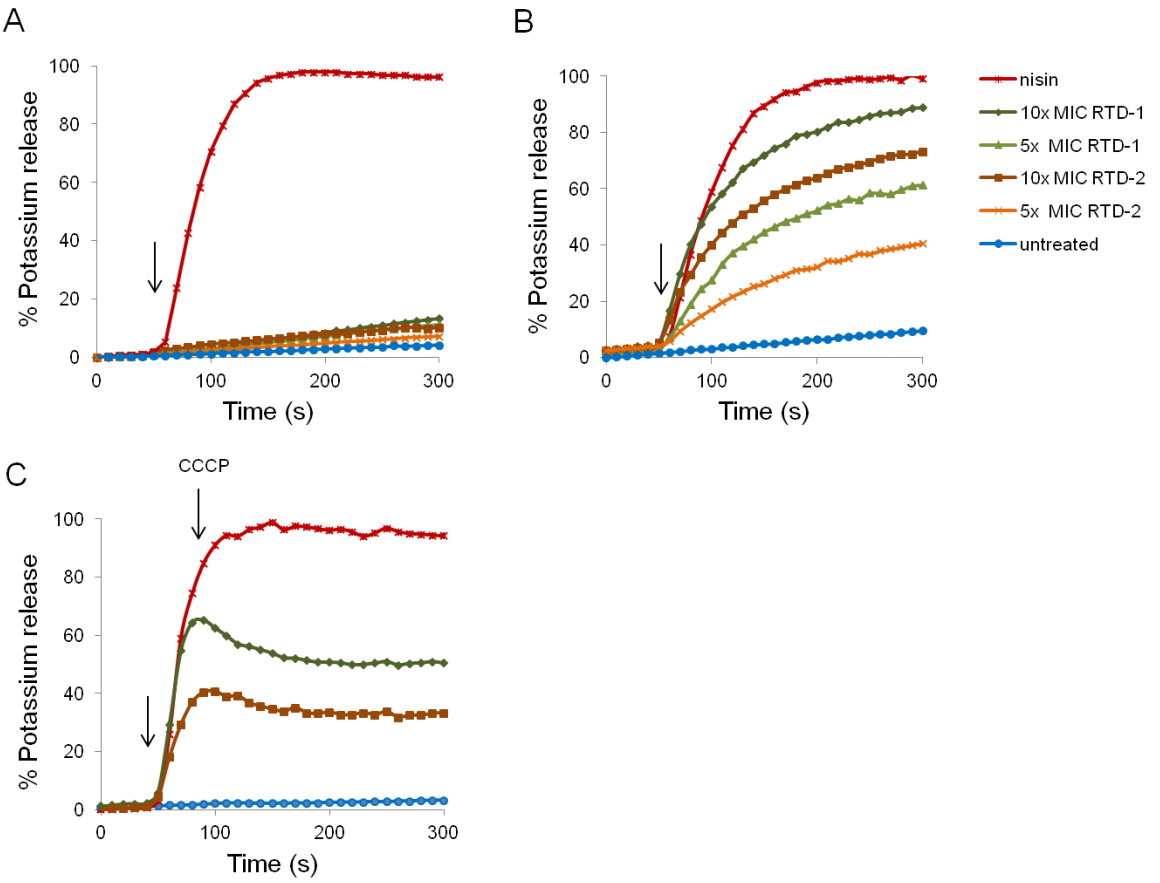

Figure 15 Impact on the membrane integrity of RTD-treated *S. simulans* 22 cells. Potassium efflux was monitored with a potassium-sensitive electrode in absence (A) and presence (B) of 10 mM glucose. The RTD-induced potassium release of energised cells could be blocked by the addition of 5 µM CCCP (carbonyl cyanide m-chlorophenylhydrazone; C).
Ion leakage was expressed relative to the total amount of potassium released after addtion of 1 µM of the pore-forming lantibiotic nisin (100% efflux). RTDs were added at 5x and 10x MIC; controls were incubated without peptide. The arrows indicate the moment of peptide addition.

These results indicate that the membrane activity of RTDs depends on the bacterial membrane potential. To further investigate this hypothesis, 5 µM of the ionophore CCCP (carbonyl cyanide m-chlorophenylhydrazone) were added to energised cells shortly after the peptides. CCCP uncouples the proton gradient across the cytoplasmic membrane leading to fast membrane depolarisation. Indeed, RTD-induced ion leakage was blocked immediately after CCCP addition (Figure 15 C).

Moreover, the membrane potential of *S. simulans* 22 in choline buffer (used for the potassium efflux experiments) was estimated by the distribution of the lipophilic cation TPP^+ inside and outside the cells. As expected, the membrane potential increased by

about 15-20 mV after incubation with 10 mM glucose (Figure 16). Thus, a high membrane potential - as it can be observed in presence of glucose - seems to be essential for the membrane-disrupting activity of RTDs.

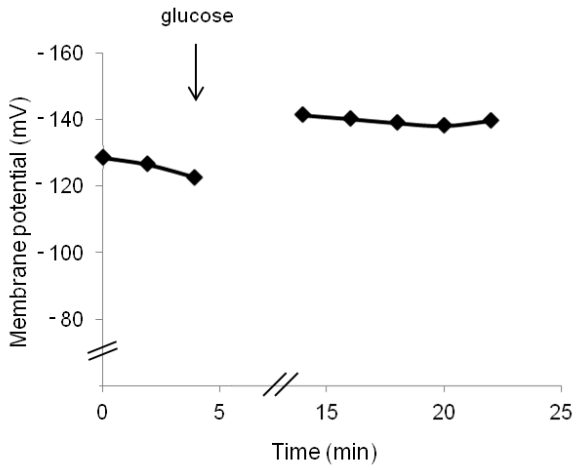

Figure 16 Membrane potential of *S. simulans* 22 suspension in choline buffer (300 mM choline chloride; 30 mM MES, 20 mM Tris; pH 6.5) used for potassium efflux experiments without an energy source. After 5 min incubation 10 mM glucose were added to the cells (arrow).

Further, the membrane potential of *S. aureus* SG511-Berlin was monitored in half-concentrated Mueller-Hinton broth which was routinely used for MIC determinations and mode of action studies. In absence of glucose, the peptides did not cause any change of the membrane potential when added at 10x MIC (Figure 17 A). In contrast, the addition of nisin induced a significant drop of the membrane potential of *S. aureus* SG511-Berlin (Figure 17 A). In presence of glucose, the membrane potential increased only slightly (approximately 10 mV; Figure 17 B). However, RTDs at 10x MIC caused a reduction of the membrane potential of about 15 mV which was completely restored after 20 min of treatment (Figure 17 B).

Altogether, the degree of membrane impairment depends on the level of energisation and therefore on the membrane potential across the bacterial membrane. In cells with a certain membrane potential, θ-defensins disrupt the membrane barrier function causing a rapid efflux of small molecules such as potassium ions and a concomitant drop of the membrane potential.

However, these data did not explain the complete cessation of all biosynthetic reactions as only a minor impact on the membrane integrity could be observed in medium lacking free glucose. Thus, the question was raised if the membrane impairment alone is sufficient for killing by RTDs or if additional activities are involved in the killing mechanism.

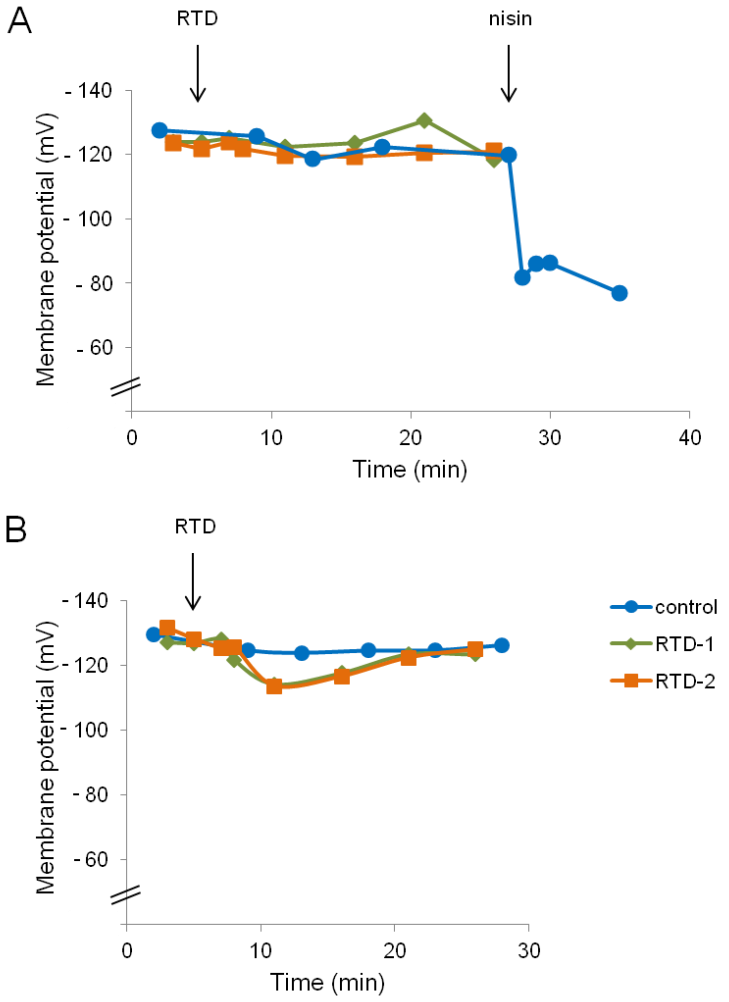

Figure 17 Membrane potential of *S. aureus* SG511-Berlin in half-concentrated Mueller-Hinton broth in absence (A) and presence (B) of 10 mM glucose. RTDs were added in concentrations corresponding to 10x MIC. The pore-forming peptide nisin (10x MIC) was used as a control. Arrows indicate the moment of peptide addition.

3.2.5 Morphological changes of RTD-2 treated *S. aureus* cells

Further, RTD-treated cells of *S. aureus* SG511-Berlin grown in half-concentrated Mueller-Hinton broth, were inspected by transmission electron microscopy in order to obtain informations on potential additional activities. After 30 min treatment, additional membranous structures could be observed (Figure 18 A-C) in many cells, indicating the loss of cytoplasmic content. After 60 min exposure to RTD-2, cells showed degradation of the cell wall, particularly in the septum area between two daughter cells (Figure 18 D, E). Moreover, in some cells, the cell wall was completely peeled off (Figure 18 F). These morphological changes might indicate a premature activation of peptidoglycan lytic enzymes (referred to as autolysins) involved in cell separation.

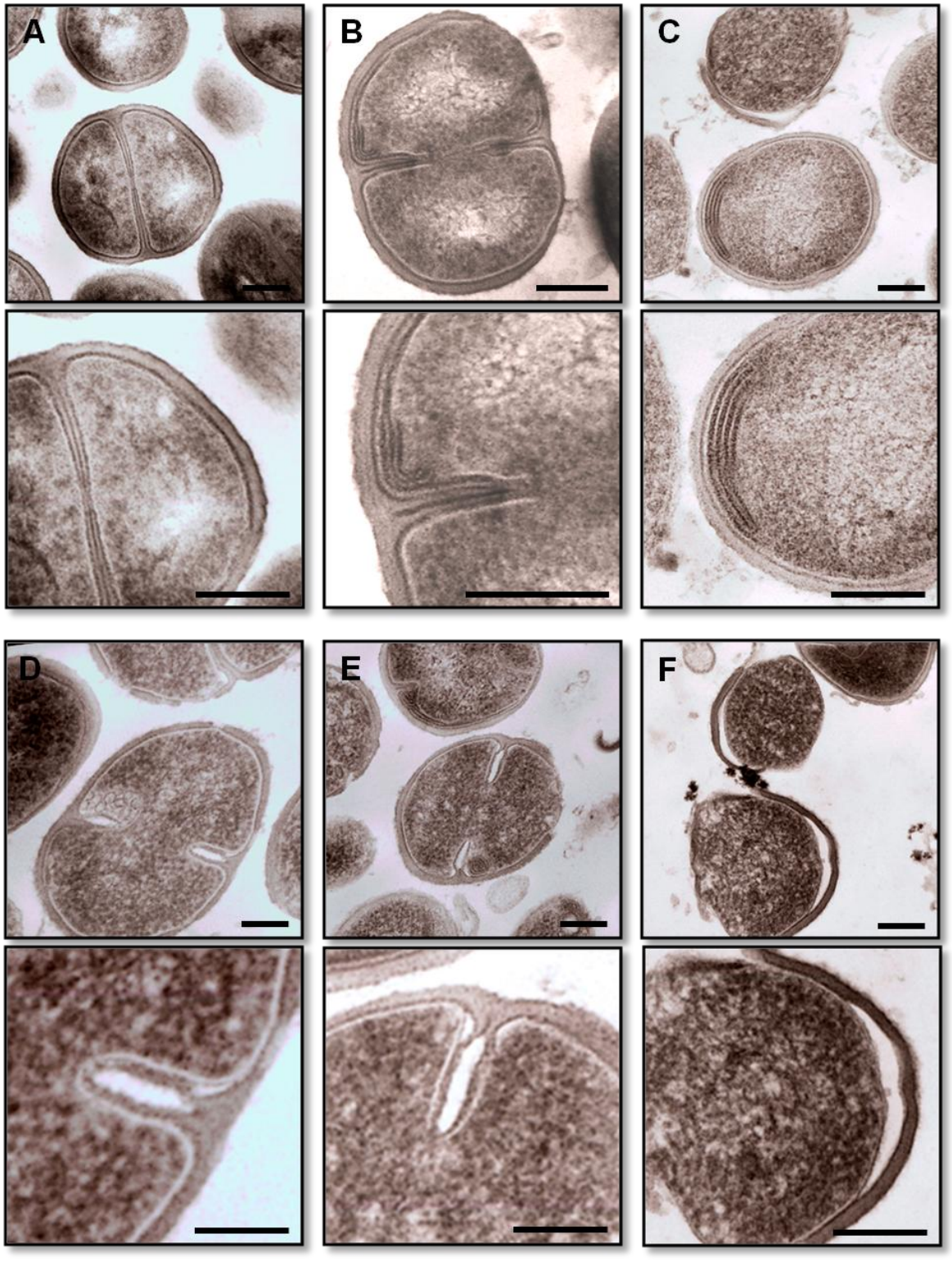

Figure 18 Transmission electron microscopy of *S. aureus* SG511-Berlin treated with 10x MIC RTD-2. A: Untreated control cells. B, C: Cells treated for 30 min. Additional membranous structures could be observed. D, E, F: Cells treated for 60 min. Dividing cells showed degradation of the cell wall in the septum area between two daughter cells (D, E) or peeling of the cell wall (F). Scale bar: 0.2 µm.

Interestingly, RTD-treated cells resemble those exposed to the cationic antimicrobial peptide Pep5 produced by *Staphylococcus epidermidi*s 5 (Bierbaum and Sahl, 1991). It has been demonstrated that Pep5 induces autolysis in *S. simulans* 22 (formerly *S. cohnii* 22) by releasing autolytic enzymes (Bierbaum and Sahl, 1985; Bierbaum and Sahl, 1987). In addition, Pep5 formes pores in energised cells leading to rapid efflux of small molecules (Sahl, 1985; Sahl and Brandis, 1983). Thus, it seems likely that the release of autolysins which hydrolyse the glycan chain and peptide bridges of murein also contributes to the killing by RTDs.

3.2.6 Release of cell wall lytic enzymes by RTDs

To provide further evidence that release of cell wall lytic enzymes is a relevant component of the antistaphylococcal activity of RTDs, the supernatant of RTD-treated cells was analysed for autolytic activity. Hence, *S. aureus* SG511-Berlin was incubated in the presence of RTDs at 10x MIC for 30 or 60 min, harvested and the concentrated supernatants were applied to a SDS-PAGE containing heat-inactivated *M. luteus* cells as substrate (compare section 2.5.3). Clear bands indicated the cell wall lytic activity of released enzymes. Cells exposed to Pep5 were included in the study and served as a positive control.

Autolysins could be observed in all peptide treated samples, whereas hardly any activity was detectable in the untreated control (Figure 19 A). Interestingly, all detected bands represent different processed forms of the autolysin Atl as in an *atl* deletion mutant (*S. aureus* SA113 Δ*atl*; Figure 19 B) corresponding bands were missing after treatment with RTD-2 at 10x MIC. Atl is a bifunctional autolysin that plays a key role in separating cells after cell division and is highly conserved among staphylococci (Albrecht et al., 2012). Proteolytic processing of the Atl precursor of *S. aureus* generates two catalytically active enzymes fused to repeat units, an amidase (AM, 62 kDa) and a glucosaminidase (GL, 51 kDa), that both bind to the septum site of dividing cells (Schlag et al., 2010; Yamada et al., 1996). Besides the AM and GL bands, three additional bands with molecular masses of 138 kDa, 113 kDa and 87 kDa could be detected. The 138 kDa band corresponded to the full length protein (Pro-Atl). The 113 kDa and 87 kDa bands presumambly represented the unprocessed amidase and glucosaminidase domain after proteolytic cleavage of the signal and propeptide (Atl) and the amidase with the propeptide (PP-AM), respectively (according to Schlag et al., 2010).

Remarkable differences could be revealed between RTD-1 and RTD-2 treated cells. A stronger enzyme activity was detected in the supernatant of cells exposed to RTD-2 - particular after 60 min treatment (Figure 19).

Recently, it has been demonstrated that the Atl amidase is directed to the septal region by its repeat domains where it binds to lipoteichoic acid (LTA) (Zoll et al., 2012). Consequently, the release of Atl by Pep5 as well as RTDs suggests that these cationic peptides bind to the polyanionic LTA, thereby replacing the autolytic enzymes and inducing autolysis.

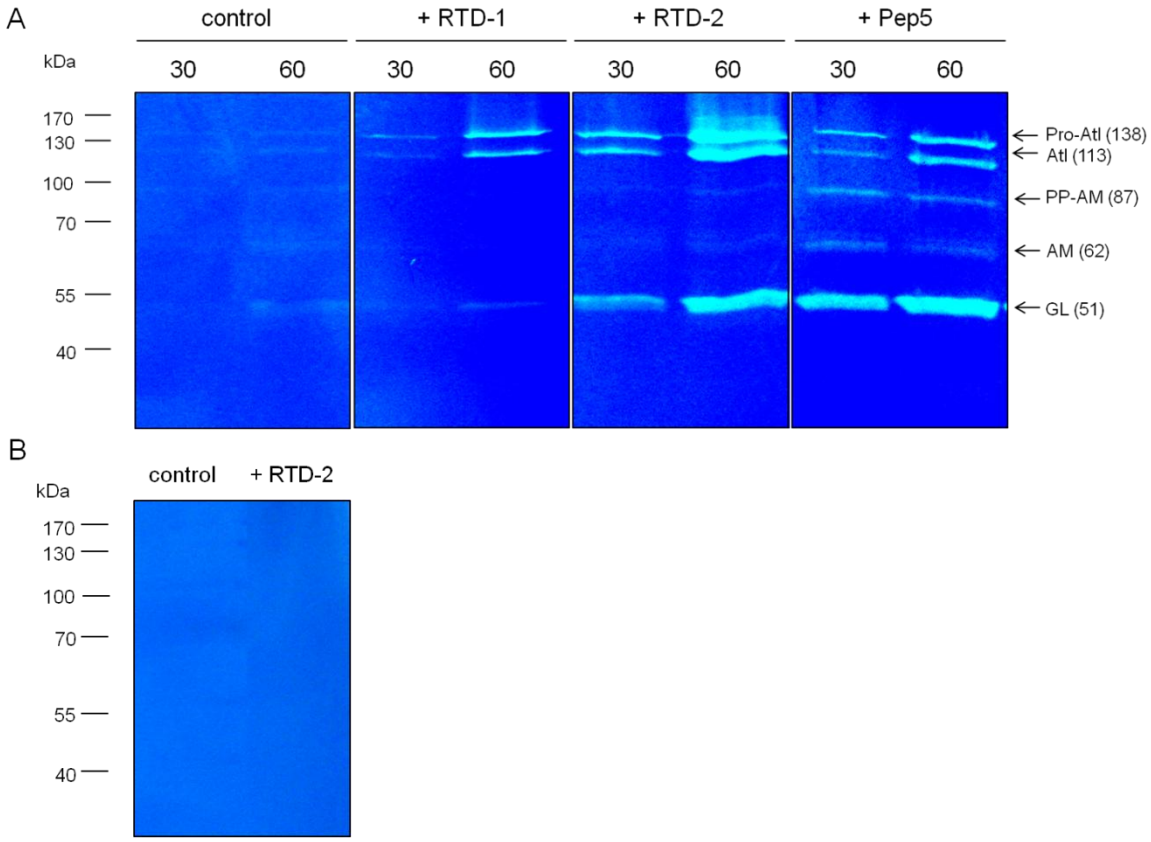

Figure 19 Detection of cell wall lytic enzymes in the supernatant of RTD-treated cells. A: *S. aureus* SG511-Berlin was exposed to 10x MIC of RTDs or Pep5 for 30 and 60 min. Equal amounts of the concentrated culture supernatant (containing autolysins released from the cell surface) were separated on a 12% SDS-PAGE containing heat-inactivated *M. luteus* cells as substrate. Clear bands represent cell lysis zones.
B: *S. aureus* SA113 Δ*atl* treated for 60 min with 10x MIC RTD-2.
All bands represent differently processed Atl forms as in the *atl* deletion mutant the corresponding autolysis bands were missing (B).
Pro-Atl: Atl with full-length propeptide, Atl: amidase and glucosaminidase, PP-AM: amidase with propeptide, AM: amidase, GL: glucosaminidase.

Consistent with the release of the autolysin Atl by RTDs, θ-defensin-mediated killing was diminished in *S. aureus* SA113 Δ*atl*. Killing kinetics of the *atl* deletion mutant and its wild-type strain showed that the mutant was significantly more resistant towards the action of RTD-2 (Figure 20). The number of colony forming units (CFU) of the WT was reduced by

several logs after addition of the peptide, whereas the *atl* deletion mutant was only slightly affected by RTD-2 even at 80 µg/ml (Figure 20).

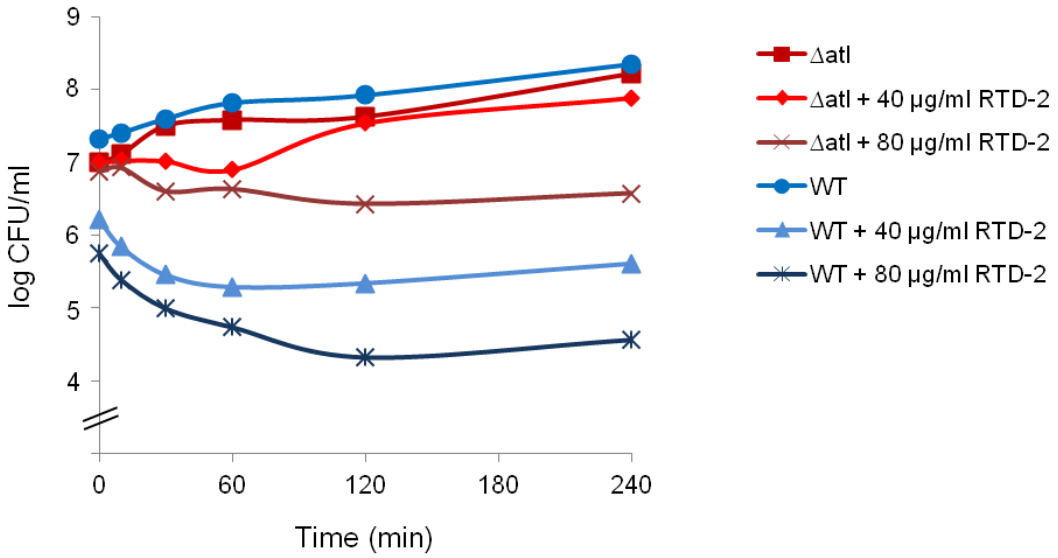

Figure 20 Killing kinetic of *S. aureus* SA113 (WT) and its *atl* deletion mutant (Δ*atl*) in half-concentrated Mueller-Hinton broth and presence of 40 and 80 µg/ml RTD-2 over a period of 4 h.

However, no differences of the MIC values between the wildtype and the mutant could be detected after 24 h incubation (Table 11) as the bacteria which have not been killed by RTDs were able to regrow during this long incubation time.

Since LTA serves as an anchor molecule for Atl (Zoll et al., 2012), two strains with an altered LTA content were included in the study. *S. aureus* SA113 Δ*ypfP* has a 87% reduced LTA content compared to the wild-type strain and the remaining LTA are directly linked to diacylglycerol (DAG) instead of diglucosyl-DAG (Glc2-DAG) (Fedtke et al., 2007). Moreover, the recently described LTA-deficient suppressor strain *S. aureus* SEJ1 Δ*ltaS* (4S5) was tested for its susceptibility towards RTDs and Pep5. Unexpectedly, both strains were more susceptible towards Pep5, while only *S. aureus* SEJ1 Δ*ltaS* (4S5) showed a lower MIC of RTDs compared to the parental strain (Table 11).

Table 11 Antibacterial activity of RTDs and Pep5 (µg/ml)* against *S. aureus* mutants affected in autolysis or LTA synthesis in 10% Mueller-Hinton broth. MIC values are expressed as the lowest concentration that caused visible growth inhibition.

Strain	RTD-1	RTD-2	Pep5
S. aureus SA113	16	8	4
S. aureus SA113 Δ*atl*	16	8	4
S. aureus SA113 Δ*ypfP*	16	16	1
S. aureus SEJ1	>32	32	4
S. aureus SEJ1 Δ*ltaS* (4S5)	4	2	0.125

*Average values obtained from two or more independent experiments.

3.2.7 Antagonisation of antibacterial activity of θ-defensins

LTA serves as a receptor for the repeat domains of AM and GL at the septum (Zoll et al., 2012) and it was proposed that RTDs and Pep5 bind to LTA, thereby releasing autolytic enzymes. To further confirm that the peptides interact with LTA, an antagonisation assay was performed. For this, isolated LTA was added as potential antagonist to a liquid growth inhibition assay in a 1:1, 1:2 or 1:4 molar ratio with respect to the peptide. Indeed, addition of LTA inhibited the antimicrobial activity of RTDs when used at 1:4 molar ratio, but did not antagonise the antibiotic action of Pep5 (Table 12). Earlier studies reported an inhibition of Pep5-induced lysis by LTA at a 10-fold molar excess (Bierbaum and Sahl, 1985).

Table 12 Antagonisation of antimicrobial activity of θ-defensins and Pep5 against *S. aureus* SG511-Berlin by LTA. The following symbols are used: + for antagonisation; - for normal antimicrobial activity.

Molar ratio peptide:LTA	RTD-1	RTD-2	Pep5
1:1	-	-	-
1:2	-	-	-
1:4	+	+	-

3.2.8 Release of carboxyfluorescein from LTA-containing liposomes

As it was proven that θ-defensins and Pep5 interact with membrane-bound teichoic acids, it was further investigated whether this interaction also facilitates the pore-formation process. For example, nisin and related peptides use lipid II as docking molecule to subsequently form pores in the membrane of susceptible strains.

Therefore, carboxyfluorescein-loaded liposomes were made of DOPC and 0.5 mol% purified LTA and the efflux of the fluorescent dye was monitored after addition of 1 µM of each peptide. Pure DOPC vesicles were used as a control. As shown in Figure 21, only minor marker release was observed with pure DOPC vesicles and DOPC doped with LTA. Thus, LTA does not seem to be involved in the membrane-disrupting activity of RTDs and Pep5.

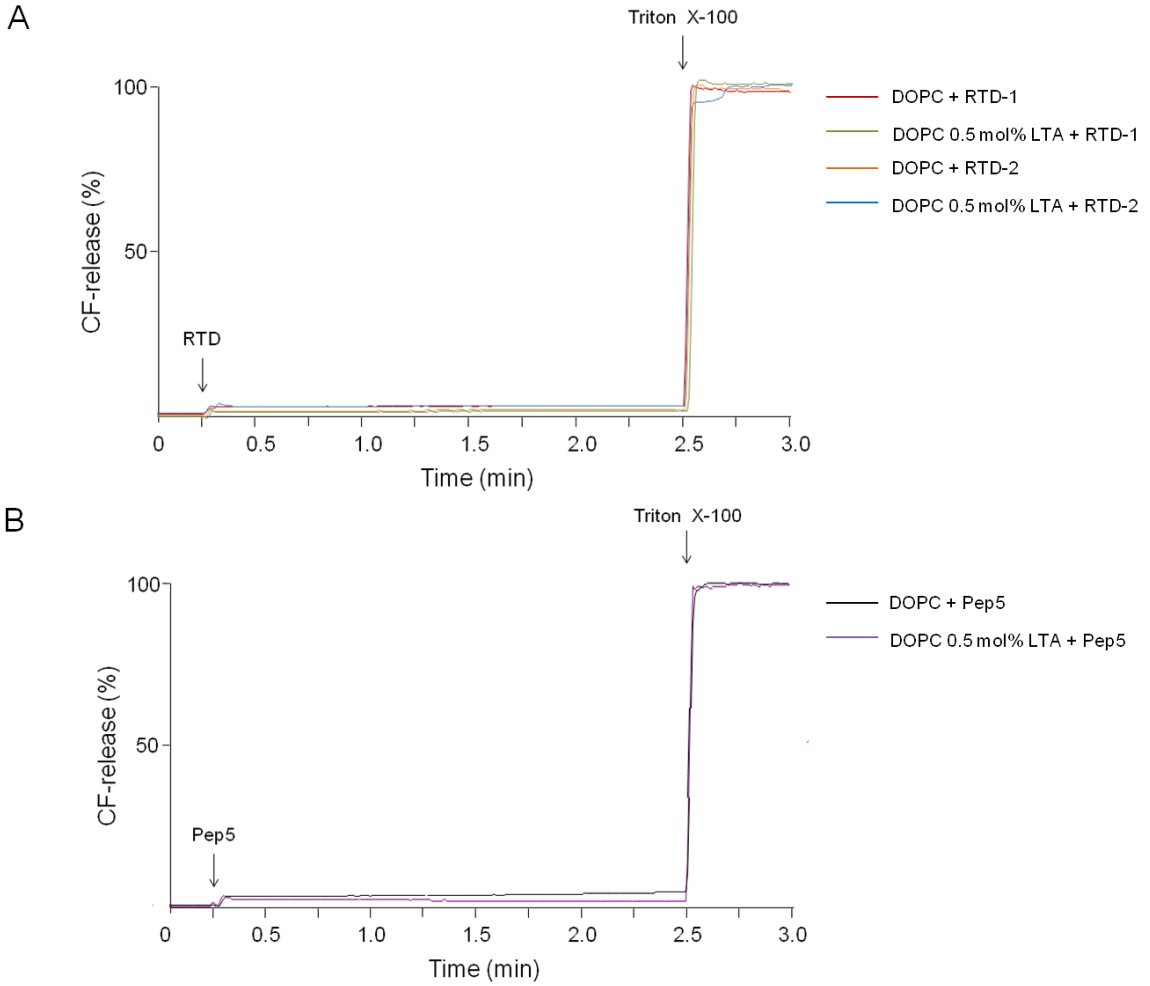

Figure 21 Carboxyfluorescein (CF) release from liposomes made of DOPC and 0.5 mol% LTA isolated from *S. aureus* SG511-Berlin. RTDs (A) and Pep5 (B) were added at 1 µM. Marker release was expressed relative to the amount of CF released after addition of Triton X-100 (100% efflux). Pure DOPC vesicles were used as a control.

3.2.9 Susceptibility testing of a PepI producing strain

The Pep5 producer strain *S. epidermidis* 5 protects itself from the lethal action of its own peptide by the immunity peptide PepI. This peptide consists of 69 amino acids forming a hydrophobic N-terminal domain and a hydrophilic C-terminus. It has been demonstrated that PepI is translocated across the membrane and localises in the outer leaflet of the cytoplasmic membrane where it probably shields the Pep5 target (Hoffmann et al., 2004). However, the interaction partner of PepI is still unknown.

The results described above indicate that RTDs may act in a similar way as the lantibiotic Pep5. In order to examine if PepI also confers resistance towards RTDs, a MIC determination with *S. carnosus* TM300 carrying *pepI* under a xylose-inducible promotor was performed. As expected, PepI expression resulted in significant Pep5 insensitivity (MIC increased by the factor 32), while the RTD sensitivity decreased by the factor 2 to

2.5 (Table 13), indicating that PepI can in part contribute to θ-defensin resistance. These results provide a further hint that Pep5 and RTDs may have a similar mechanism of action.

Table 13 MIC values (µg/ml)* of *S. carnosus* TM300 expressing *pepI* under a xylose-inducible promotor in half-concentrated Mueller-Hinton broth. PepI synthesis was induced by addition of 1% xylose. MIC values are expressed as the lowest concentration that caused visible growth inhibition.

	RTD-1	**RTD-2**	**Pep5**
***S. carnosus* TM300 pAH-PepI**			
- xylose	1	0.75	0.06
+ xylose	2	2	2

*Average values obtained from two or more independent experiments.

3.3 Part 3: Insight into the Gram-negative mode of action of defensins

The epithelial human β-defensin 3 (hBD3) is a highly cationic peptide (net charge +11) that forms dimers in solution (Schibli et al., 2002) and retains antimicrobial activity at physiological salt concentrations (Boniotto et al., 2003; Harder et al., 2001). Harder et al., (2001) first described hBD3 and observed cell wall perforations of hBD3 treated staphylococcal cells, indicating interference of hBD3 with peptidoglycan biosynthesis or localised induction of lytic enzymes. Later studies confirmed that hBD3 bind to the cell wall precursor lipid II in a 1:1 molar ratio (Sass et al., 2010). Further, hBD3 causes a small, but significant reduction of the membrane potential and the transcriptional response pattern of hBD3-treated staphylococci is in part similar to that of cells exposed to membrane-active α-helical HDPs, suggesting that hBD3 additionally affects energy generation and membrane transport processes. Thus, killing of *S. aureus* by hBD3 is the result of pleiotropic effects on membrane bound processes on top of specific cell wall inhibition caused by lipid II sequestration (Sass et al., 2008; Sass et al., 2010).
In addition, the peptide displays activity against Gram-negative bacteria and fungi (Harder et al., 2001; Garcia et al., 2001). It has been proposed – after analysis of chimeric and shortened hBD3 peptides – that the antibiotic action against *S. aureus* is linked to the N-terminus whereas the highly charged C-terminal part plays a predominant role in the activity against *E. coli* (Hoover et al., 2003; Jung et al., 2011). Here, hBD3 was analysed for its activity against Gram-negative bacteria by studying its interaction with the cell envelope of *E. coli*.

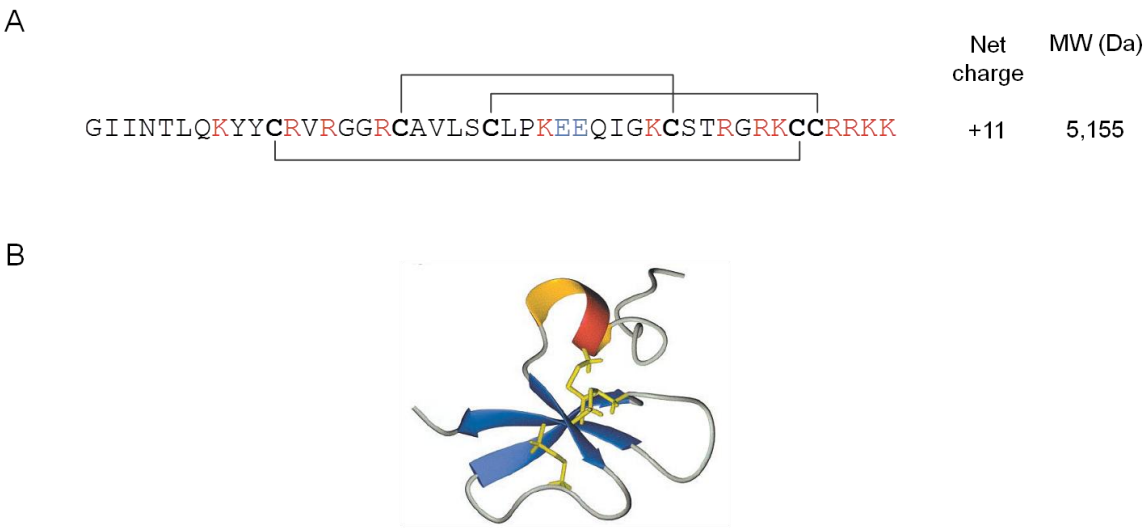

Figure 22 A: Amino acid sequence, molecular weight and net charge of hBD3 (at pH 7). Residues with a positive charge are marked in red, negatively charged residues are marked in blue. B: 3D structure of hBD3 (Schibli et al., 2002).

3.3.1 Antibacterial activity of hBD3 against *E. coli*

Initially, the antimicrobial activity of hBD3 against different *E. coli* strains was determined in 10% Mueller-Hinton broth which was used as medium in all further experiments. Under this condition, the defensin inhibited the growth of all three test strains at a concentration of 1 µM (Table 14).

Table 14 Antibacterial activity of hBD3 against different *E. coli* strains in 10% Mueller-Hinton broth. MIC values (µM)* are expressed as the lowest concentration that caused visible growth inhibition.

Strain	MIC (µM)
E. coli ATCC 25992	1
E. coli BW25113	1
E. coli ML-35pYC	1

*Average values obtained from two or more independent experiments.

3.3.2 Growth kinetic measurements in presence of hBD3

Growth kinetic measurements of *E. coli* BW25113 exposed to hBD3 and various antibiotics with known cellular targets (at 5x MIC) were carried out in order to obtain information on its mode of action against Gram-negative bacteria. Interestingly, the hBD3 behaviour was neither similar to the rapid membranolytic action of polymyxin B nor to the activity of cell wall inhibitors (ampicillin, penicillin G; Figure 23) which cause killing over the course of one bacterial generation.

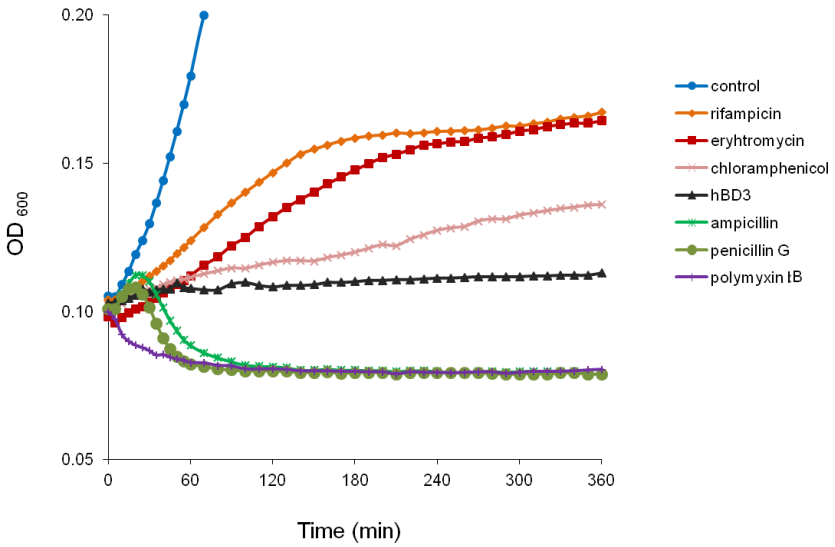

Figure 23 Growth kinetic measurements of *E. coli* BW25113 in presence of hBD3 and various antibiotics with known cellular targets at 5x MIC.

In contrast, hBD3 induced cellular lysis in *S. aureus* SG511-Berlin which is consistent with the reported inhibition of cell wall biosynthesis (data not shown).
These results suggest that the activity of hBD3 against Gram-negative bacteria differs from its mode of action against Gram-positive bacteria and that additional targets might be involved in killing.

3.3.3 Permeabilisation of *E. coli* outer and inner membrane

The ability of hBD3 to permeabilise both the outer and the cytoplasmic membrane of *E. coli* ML-35pYC was studied by following the hydrolysis of two extracellular chromogenic reporter molecules. Outer membrane disruption was indicated by cleavage of the cephalosporin CENTA^{TM} by the periplasmic β-lactamase of this strain (Figure 24 A), whereas inner membrane permeabilisation was measured by the rate of ONPG (o-nitrophenyl-β-D-galactopyranoside) hydrolysis by the cytoplasmic β-galactosidase (Figure 24 B). Polymyxin B – which is known to induce pore-formation in both membranes – was used as a positive control.
A rapid hydrolysis of CENTA^{TM} was observed in presence of polymyxin B and hBD3, indicating efficient outer membrane disruption. At 5x MIC, hBD3 achieved similar levels of membrane permeabilisation as polymyxin B (Figure 24 A).
In contrast, inner membrane permeabilisation was significantly slower in cells exposed to hBD3 in comparison to those treated with polymyxin B. Interestingly, no further ONPG hydrolysis could be measured after 60 min treatment with 5x MIC of hBD3, although the maximum reaction rate had not been reached (Figure 24 B).

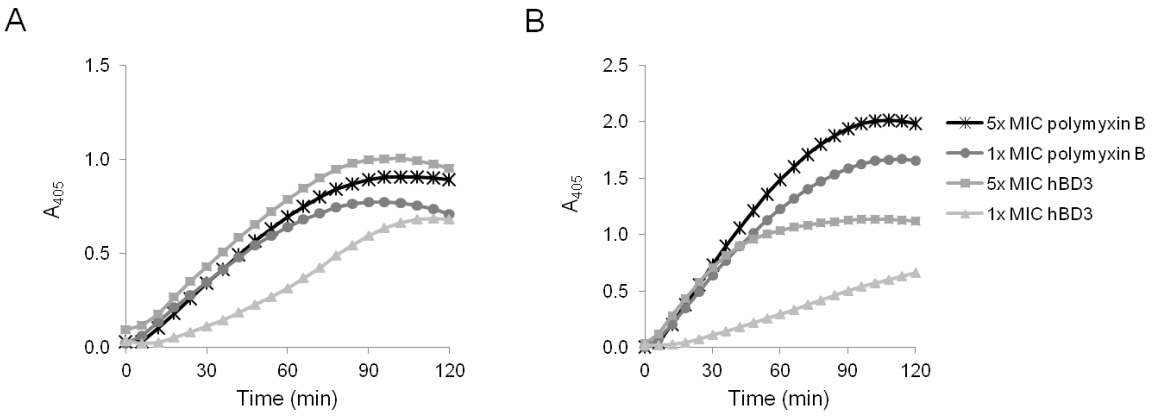

Figure 24 Inner and outer membrane permeabilisation of *E. coli* ML35-pYC. A: Outer membrane permeabilisation was assessed by the hydrolysis of the extracellular chromogenic cephalosporin CENTA^{TM} by the periplasmic β-lactamase of this strain. B: Inner membrane permeabilisation was determined by following the cleavage of the extracellular substrate ONPG by the constitutively expressed β-galactosidase of *E. coli* ML35-pYC. The pore-forming antibiotic polymyxin B was used as a control.

3.3.4 Morphological changes of hBD3 treated *E. coli* cells

To further investigate the effect of hBD3 on *E. coli* BW25113, the morphology of treated cells was examined by transmission electron microscopy. Blebbing of the outer membrane was noted within 30 min of exposure (Figure 25 B, C) and became more frequent after 60 min (Figure 25 D, E). This effect is indicative of LPS released from the cell surface. Moreover, densed material could be monitored on the external face of the outer membrane of some cells (Figure 25 B, E).

Consistent with the previous described results (Figure 23), the interaction of hBD3 with the membranes of *E. coli* did not cause a significant loss of cytoplasmic content that would indicate generalised cell rupture.

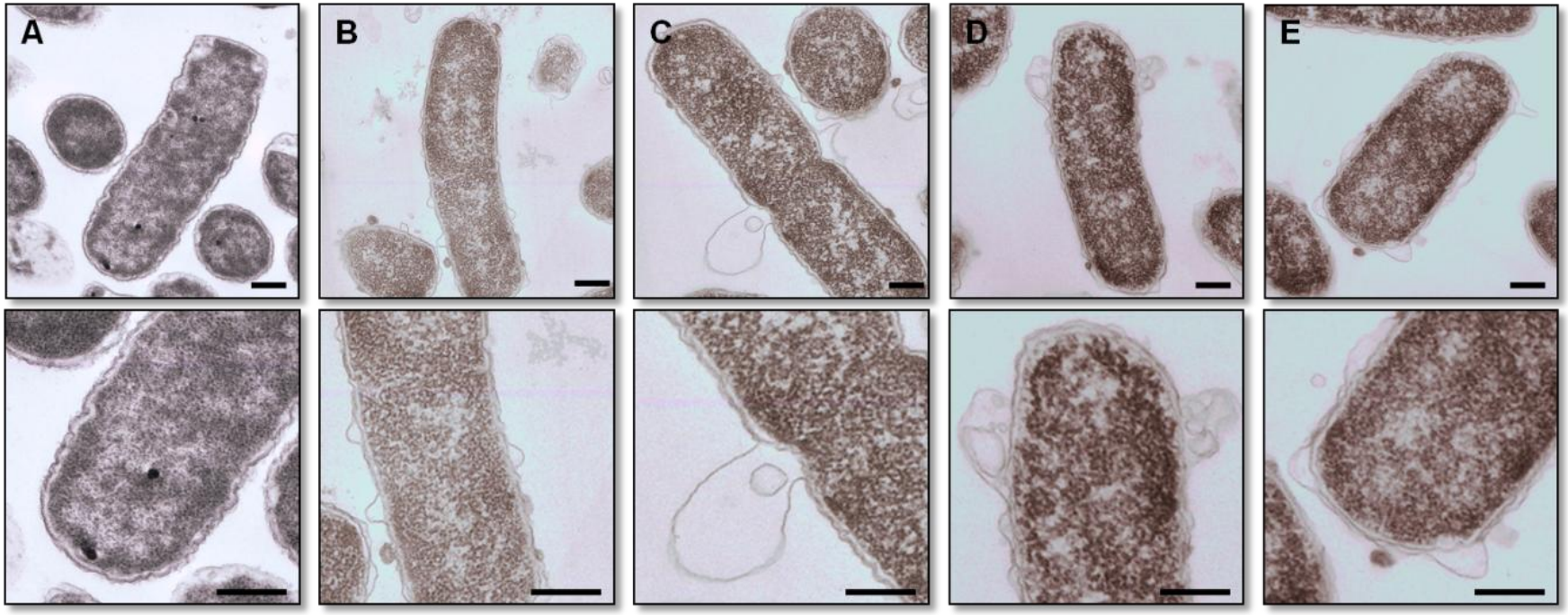

Figure 25 Transmission electron microscopy of *E.coli* BW25113 treated with 10x MIC hBD3. A: Untreated control cells. B, C: Cells treated for 30 min. D, E: Cells treated for 60 min. Evaginations of the outer membrane could be observed. Scale bar: 0.4 µm.

3.3.5 Activity of hBD3 against *E. coli* strains with different LPS structures

As the initial interaction of hBD3 with the outer membrane seems to be crucial for its activity, the susceptibility of *E. coli* strains with different LPS structures was investigated by determining the IC_{50} values (Figure 26 B). Among these strains, *E. coli* ATCC 25992 with full-length LPS and *E. coli* BW25113 lacking the O antigen were significantly more susceptible towards hBD3 than the tested mutants which differ in their LPS core region (Figure 26).

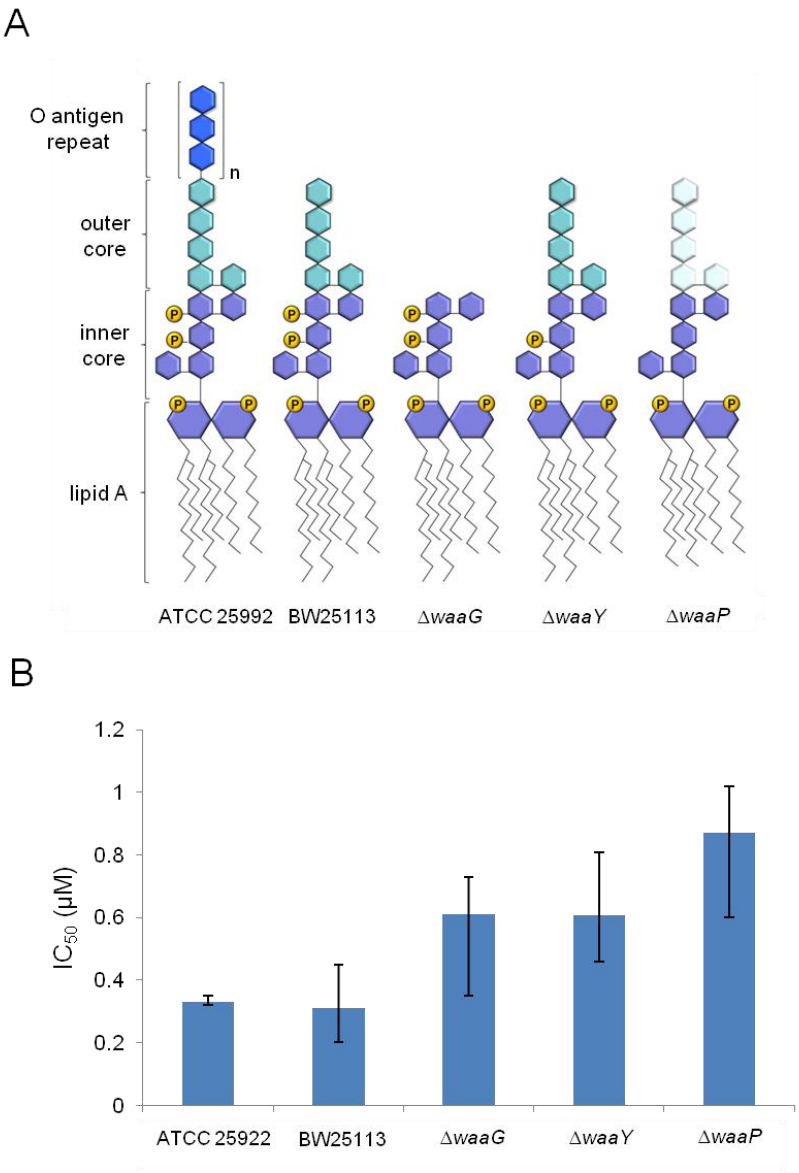

Figure 26 A: LPS structures of different *E. coli* strains tested. ATCC 25992: full-length LPS, BW25113: lacks the O antigen, Δ*waaG*: lacks the outer core region, Δ*waaY*: lacks one phosphate group in the inner core region, Δ*waaP*: deficient in core phosphate.
B: IC_{50} values of hBD3 (µM) against the *E. coli* strains described above.

The kinases WaaP and WaaY are involved in phosphorylation of heptose residues in the inner core region of LPS. Thus, a mutation in one of the corresponding genes decreases the net negative charge of the LPS molecules, and thereby lowering the affinity towards cationic peptides. Consistently, the Δ*waaP* and Δ*waaY* deletion mutants were more resistant towards hBD3. Interestingly, *E. coli* Δ*waaG* lacking the outer core region showed also a higher hBD3 resistance compared to the wild-type strain (*E. coli* BW25113).
Hence, the susceptibility towards hBD3 depends on the composition of LPS, and not only the phosphate residues, but also the sugars of the outer core region seem to be involved in the interaction.

4 Discussion

The antibacterial mode of action of defensins was long thought to result from electrostatic interaction between the cationic peptides and the negatively charged microbial membranes, followed by pore-formation or unspecific membrane permeabilisation. Previous studies and the results of this thesis have clearly demonstrated that the action of defensins can be much more targeted and that specific lipid-bound molecules of the microbial cell envelope are involved in killing.

4.1 Part 1: The central cell wall building block lipid II as target for defensins

Here, the mode of action of antibacterial invertebrate defensins was studied with three defensin variants (*Cg*-Defm, *Cg*-Defh1, *Cg*-Defh2) isolated from the oyster *C. gigas*. Results showed that oyster defensins kill *S. aureus* through binding to lipid II, thus inhibiting peptidoglycan biosynthesis. To date, only invertebrate defensins with antifungal activity had been characterised in terms of cellular targets (Thevissen et al., 2004).

The biosynthesis of bacterial cell wall peptidoglycan is a multistep process involving reactions in the cytoplasm and both sides of the cytoplasmic membrane (for details see Figure 27). It is a prominent target for many antibacterial compounds from different chemical classes, including glycopeptides, lipopeptides and lantibiotics. Particularly, the building block lipid II is trapped by many compounds when presented on the outer face of the bacterial cytoplasmic membrane. For example, the clinically-used glycopeptides such as vancomycin bind to the D-Ala-D-Ala terminus of the lipid II side chain, thereby causing sequestration of the precursor from PBPs and subsequently blocking its further incorporation into the cell wall (Schneider and Sahl, 2010). Moreover, the lantibiotic nisin (which was used as a positive control during this study) and related peptides combine lipid II targeting with membrane permeabilisation (Wiedemann et al., 2001). Very recently, lipid II binding has also been described for one fungal defensin named plectasin (Schneider et al., 2010) and two mammalian defensins – representatives of the α- and β-defensin family – namely HNP-1 and hBD3 (de Leeuw et al., 2010; Sass et al., 2010).

The oyster defensins displayed potent activity against several Gram-positive bacteria, including staphylococci which were chosen for further mode of action studies (Table 8).

Incubation of *S. simulans* 22 with oyster defensins resulted in the accumulation of the last soluble cell wall precursor UDP-MurNAc-pp in the cytoplasm, comparable to classical inhibitors of the membrane-bound steps of cell wall biosynthesis (Figure 8). Moreover, lipid II antagonised the oyster defensin activity against *S. aureus*, whereas C_{55}-P (which serves as a lipid carrier in lipid II) as well as UDP-MurNAc-pp and UDP-GlcNAc (the

activated cell wall sugars forming the disaccharide moiety of lipid II) did not (Table 9). The antagonisation of the oyster defensins antibacterial activity by lipid II occurred at a 1:1 molar ratio (Table 9), as also observed for plectasin (Schneider et al., 2010) and for hBD3. However, the latter one was also antagonised by C_{55}-P and negatively charged phospholipids when added in up to a 5-fold molar excess (Sass et al., 2010). The 1:1 stoichiometry was also confirmed here by TLC, as indicated by the absence of free lipid II on the plate at this molar ratio (Figure 10).

Altogether, this result suggests that targeting of lipid II by oyster defensins requires lipid II-specific molecular determinants such as the disaccharide-pyrophosphate moiety, which is targeted by the lantibiotics. NMR-based modelling of the plectasin-lipid II complex revealed that the defensin interacts with the pyrophosphate moiety of lipid II via hydrogen bonding (involving residues F2, G3, C4 and C37), whereas the hydrophobic part of the peptide is located on the membrane surface (Figure 4) (Schneider et al., 2010). Indeed, the three-dimensional structure of the oyster defensins is similar to that of plectasin although they possess a fourth disulphide bridge. Further, the amino acid residues involved in lipid II binding of plectasin are also present in the *Cg*-Defs (F2, G3, C4, C34) and other antibacterial invertebrate defensins, suggesting a conserved interaction motif. Consequently, it can be proposed that those defensins carrying a CSαβ motif and displaying exclusively antibacterial activity could have a similar mechanism of action, which involves lipid II-binding and subsequent inhibition of cell wall biosynthesis. This is consistent with their preferential activity against Gram-positive bacteria (Bulet and Stocklin, 2005; Mygind et al., 2005; Froy and Gurevitz, 2003; Otvos, 2000). In addition, antifungal invertebrate defensins with a CSαβ fold such as heliomicin do not contain the amino acids of the lipid II binding motif. Consistently, it has been shown that heliomicin does not bind to lipid II (Schneider et al., 2010) and lacks antibacterial activity (Lamberty et al., 1999), thus reinforcing the hypothesis that lipid II-binding is an essential determinant of invertebrate defensin antibacterial activity.

In contrast, antifungal invertebrate and plant defensins interact with specific sphingolipids, thereby leading to fungal cell death (compare section 1.3) (Aerts et al., 2009; Thevissen et al., 2004). Thus, evidence is increasing that invertebrate defensins, and more generally CSαβ-type defensins, are rather specific inhibitors of microbial biosynthesis or signalling pathways than mere membrane active agents.

The binding of oyster defensins to lipid II was shown to be very strong as determined here by SPR. The oyster defensins bound to DOPC vesicles containing 0.8 mol% lipid II in an almost irreversible manner, as indicated by the absence of decay in the RU values during the dissociation step (Figure 11). All defensin variants (*Cg*-Defm, *Cg*-Defh1, and *Cg*-Defh2) gave higher binding responses than nisin. Among the oyster defensins, *Cg*-Defh2

showed the highest affinity for lipid II (Figure 11). This correlates with its antimicrobial activity as *Cg*-Defh2 exhibited 2- to 4-fold lower MIC values than *Cg*-Defh1 and *Cg*-Defm (Table 8).

Interestingly, it has been demonstrated that all three defensins belong to distinct groups which evolved through gene duplication and subsequent functional divergence (Schmitt et al., 2010). This study supports the hypothesis that strong selective pressures have directed oyster defensins towards the design of new variants with higher potency. *Cg*-Defm and *Cg*-Defh2, the more active forms, exhibit a positively charged residue at position 16 (K and R, respectively; Figure 6) instead of an uncharged glutamine in *Cg*-Defh1. As these residues are exposed on the surface of the peptide (Gueguen et al., 2006) they might be involved in binding to lipid II, thus explaining the different affinity of the three variants for the peptidoglycan precursor. Similarly, a lysine on the surface of plectasin is crucial for the antibacterial activity, probably by promoting a better binding to the cell wall and membrane of bacteria (Schneider et al., 2010). Therefore, variations in oyster defensin potency could depend on charge distribution driven by sites under diversifying selection.

The binding of oyster defensins to lipid II likely occurs at the outer leaflet of the cytoplasmic membrane. At lethal concentrations (10x MIC), oyster defensins did not compromise the cytoplasmic membrane of *S. aureus* (Figure 7). For this reason, they should have no access to the inner leaflet of the cytoplasmic membrane where lipid II is synthesised and decorated with a pentaglycine interpeptide bridge before it is translocated across the cytoplasmic membrane. Consequently, the mechanism of action of oyster defensins might involve binding and sequestration of extracellular lipid II, thereby blocking its polymerisation into mature peptidoglycan (Figure 27).

Consistent with the use of lipid II as a molecular target, oyster defensins have been shown to be highly active against Gram-positive bacteria (in the nanomolar range) and barely active (≥ 20 µM) against Gram-negative bacteria (Table 8). Indeed, in Gram-negative bacteria the peptidoglycan is protected by the outer membrane preventing access of oyster defensins to the periplasmic space in the absence of membrane damage. As a consequence, the different susceptibility of Gram-positive and Gram-negative bacteria to oyster defensins probably results from a differential access to lipid II. The cell wall building block is readily accessible in Gram-positive bacteria, but requires outer membrane damage to become accessible in Gram-negative bacteria. Such outer membrane damage can be induced by high concentrations of oyster defensins, as observed above 10 µM against the Gram-negative oyster pathogen *V. splendidus* LGP32 (Duperthuy et al., 2010). Consistently, *Cg*-Defs have a synergistic effect with *Cg*-Prps (proline rich HDPs of *C. gigas*) and *Cg*-BPI (bactericidal/permeability-increasing protein of *C. gigas*) against

Gram-negative bacteria, probably by combining membrane-disrupting activities with specific lipid II binding (Gueguen et al., 2009; Schmitt et al., 2012a).

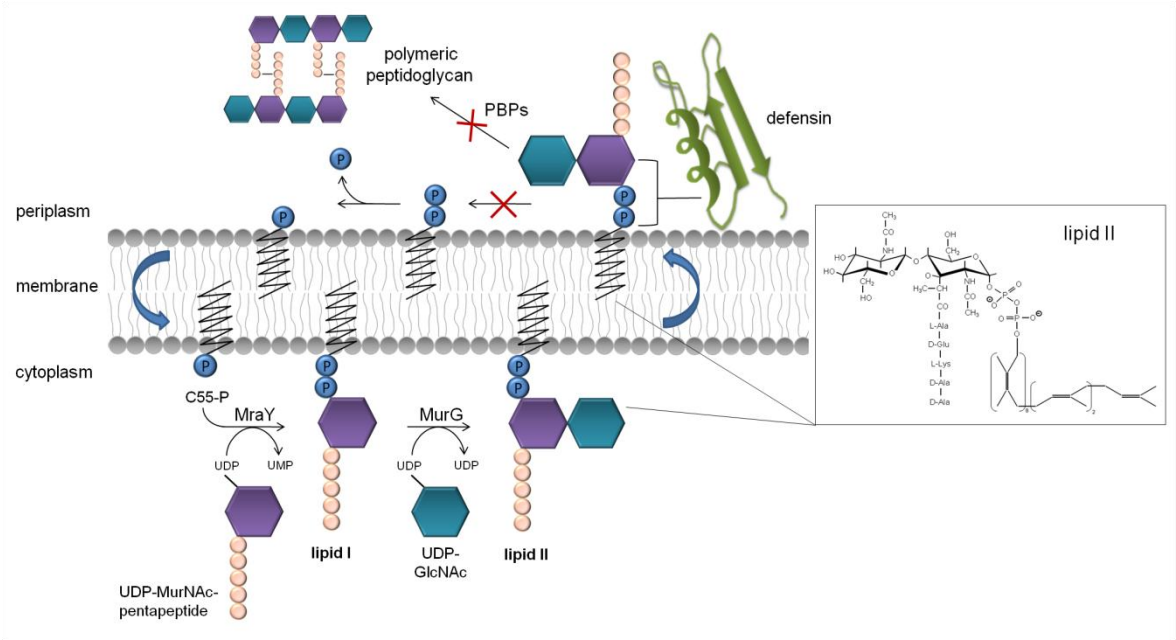

Figure 27 Inhibition of cell wall biosynthesis by defensins. Various defensins bind to lipid II, thereby causing sequestration of the precursor from PBPs and subsequently blocking its further incorporation into the cell wall.

Lipid II targeting has also been reported for the mammalian α-defensin HNP-1 (de Leeuw et al., 2010) and human β-defensin 3 (Sass et al., 2010). However, unlike invertebrate defensins, mammalian defensins are active against both Gram-positive and Gram-negative bacteria (Harder et al., 2007; Schneider et al., 2005). If both mammalian and invertebrate antibacterial defensins have the same target, what makes the invertebrate defensins so selective towards Gram-positive bacteria? One hypothesis is that unlike invertebrate antibacterial defensins, which require lipid II to be readily accessible, mammalian defensins could use their membrane-disrupting properties (Boniotto et al., 2003; Lehrer et al., 1989) to gain access to lipid II in Gram-negative bacteria. Indeed, the membrane-disrupting activity of invertebrate defensins appears much weaker than that of mammalian defensins. This was shown for oyster defensins (Figure 7), and earlier for the *D. melanogaster* defensin A, which required very high defensin/bacterial cell ratio (105:1) to induce potassium efflux in *M. luteus* (Cociancich et al., 1993). Permeabilisation of the outer membrane by cationic peptides is proposed to occur by self-promoted uptake. The initial step in this process is the interaction of the peptides with the negatively charged LPS, thereby leading to displacement of divalent cations that crossbridge adjacent LPS

molecules (Hancock, 1997). Subsequently, outer membrane disruption might depend on the cationic net charge of the peptides. For example, hBD3 displays a net charge of +11 while plectasin or the oyster defensins carrying a net charge of +1 to +3. Moreover, many vertebrate defensins form stable dimers or oligomers in solution, which results in an even higher charge density that could likely be important for their membrane activity (Boniotto et al., 2003; Schibli et al., 2002).

However, it has been demonstrated that the binding constant of plectasin to lipid II (1.8×10^{-7} M) (Schneider et al., 2010) is about one order of magnitude higher compared to the α-defensin HNP-1 (2.19×10^{-6} M) (de Leeuw et al., 2010). These findings indicate that the specificity of lipid II binding correlates to some extent with the antimicrobial spectrum. Possibly, peptides such as HNP-1 and hBD3 displaying lower affinity towards the cell wall precursor and a broader activity spectrum, may thereby retain affinity for additional targets to kill other microbes, e.g. Gram-negative bacteria (compare section 4.3).

As *Cg*-defs are only active against Gram-positive bacteria, the oyster *C. gigas* has to produce additional HDPs to control pathogens such as Gram-negative bacteria. Indeed, three representatives of the big defensin family have been recently isolated from this invertebrate (*Cg*-BigDef 1-3) (Rosa et al., 2011) which were reported to be active against both Gram-positive and Gram-negative bacteria (compare section 1.2). Moreover, two *Cg*-BPIs (bactericidal/permeability-increasing proteins) have been identified displaying exclusively Gram-negative activity (Gonzalez et al., 2007b; Zhang et al., 2011) as well as two proline-rich peptides (*Cg*-Prps) exhibiting only weak antimicrobial action (Gueguen et al., 2009; Schmitt et al., 2012a). As described above, these peptides can act synergistically, thereby increasing their potency as well as their activity spectrum (Schmitt et al., 2012a).

However, this raises the question which selective pressure has driven the development of defensins from peptides with a highly specific target to peptides with a reduced affinity and a broad-spectrum activity (as they can be found in vertebrates)? In this context, it is worth to recall that vertebrate defensins also display a diverse range of immunomodulatory functions and even bridge the innate and adaptive immune response, thereby contributing to bacterial clearance (section 1.4). In contrast, little is known about such functions in invertebrates. It has been shown that some invertebrate defensins neutralise LPS or induce signal transduction or gene transcription in mouse models (Hancock et al., 2006), but nothing has been reported about the immunomodulatory activities in the invertebrate host itself.

Nevertheless, it could be hypothesised that during the process of gaining new functions in terms of immunomodulation, defensins may have reduced their direct antimicrobial activity

and/or target specificity. This is consistent with the fact that some defensins completely lack direct antibiotic activities such as the human enteric α-defensin HD6. It has been recently found that HD6 entraps bacteria in peptide nanosets without affecting their viability (Chu et al., 2012; Ouellette and Selsted, 2012).

4.2 Part 2: Induction of autolysis as mode of action of defensins

Apparently, θ-defensins do not only differ structurally from all other defensins, they also seem to act by a different mechanism. In this study, it was demonstrated that RTDs (rhesus macaque θ-defensins) do not interfere with peptidoglycan biosynthesis (Figure 13), but rather induce bacterial lysis in staphylococci by interaction with the bacterial membrane and/or release of autolytic enzymes (Figure 28).

The membrane permeabilisation by RTDs depended strongly on the energisation of the membrane as revealed by potassium efflux experiments and membrane potential measurements (section 3.2.4). Similar observations have been described for the lantibiotic Pep5, a cationic peptide of bacterial origin. Pep5 also forms transient pores in energised membranes leading to rapid efflux of low molecular weight substances from the cytoplasm (Poppinga, 2007; Sahl, 1985; Sahl and Brandis, 1983).

Different models have been elaborated to describe the interaction of cationic peptides with the bacterial membrane. The “wedge model” was suggested to explain the pore formation process in presence of a high transmembrane potential. In this model, the amphiphilic molecules adhere to the anionic lipids on the membrane surface; in response to the electrical potential the lipid-associated peptides change the orientation (to a wegde-shape), thereby moving through the membrane to form a water-filled pore (Driessen et al., 1995; Sahl and Bierbaum, 1998). Unlike other HDPs, the highly flexible RTD structure lacks the typical amphiphilic character, even though it exhibits a certain clustering of positive charges (Trabi et al., 2001). Consequently, the peptides do not have a hydrophobic patch which allows insertion into the bilayer. In contrast, reports on the biophysical behaviour of RTD-1 in model membranes showed that the peptide induces membrane thinning when it binds to the membrane surface (Weiss et al., 2002) and that RTD molecules probably aggregate in the membrane (Buffy et al., 2004).

For Pep5, it has been hypothesised that it uses a docking molecule for binding to the cytoplasmic membrane and subsequent pore-formation (Brotz et al., 1998; Hoffmann et al., 2004). Earlier studies have demonstrated that Pep5 does not form a complex with lipid II (Brotz et al., 1998) in contrast to nisin which uses the cell wall precursor as an anchor for defined pore-formation (Wiedemann et al., 2001). Since Pep5 and RTDs bind to the membrane-anchored LTA (Table 12) (Bierbaum and Sahl, 1985; Bierbaum and

Sahl, 1987) thereby releasing autolytic enzymes, it was investigated whether this interaction also facilitates pore-formation. Under the tested conditions, neither Pep5 nor RTDs caused leakage of small vesicles containing purified LTA (Figure 21). This suggests that the membrane-disrupting activity of these peptides might depend on the membrane potential only. Voltage-dependent channel formation in model membranes has also been described for the α-defensin HNP-1 (Kagan et al., 1990).

The membrane perturbation may contribute, but not solely account for killing of staphylococcal cells by RTDs. Further, it could be demonstrated that these peptides activate peptidoglycan hydrolases (also referred to as autolysins; Figure 19). These enzymes are responsible for splitting the septum during cell division and enable incorporation of newly synthesised cell wall precursors during cell growth (Vollmer et al., 2008). The premature and uncontrolled activity of autolysins can cause cell lysis and subsequently cell death as observed in presence of θ-defensins. Zymogram analysis revealed that RTDs release differently processed forms of Atl, the most prominent autolysin of staphylococci (Figure 19). Consistently, an *atl* deletion mutant was significantly more resistant towards the activity of RTDs (Figure 20). Atl can also be activated by high salt concentrations (Heilmann et al., 1997) and the lantibiotics nisin and Pep5 (Bierbaum and Sahl, 1985; Bierbaum and Sahl, 1987). This hydrolase is synthesised as a pre-pro-peptide that is cleaved into two enzymatically active domains – an amidase (AM; that cleaves the amide bond between MurNAc and L-alanine) and a glucosaminidase (GL; that cleaves the β-1,4-gycosidic bond between GlcNAc and adjacent monosaccharides) – by an as yet uncharacterised mechanism (Heilmann et al., 1997; Schlag et al., 2010). Both enzymes localise at the cross wall of dividing cells (Yamada et al., 1996). Indeed, electron micrographs revealed that degradation of peptidoglycan of RTD-2 treated cells occurred particular at the septum between two daughter cells (Figure 18 D, E).

The AM and GL are each fused to repeat units that are responsible for targeting the enzymes to the cross wall (Baba and Schneewind, 1998; Komatsuzawa et al., 1997). It has been reported that in *S. aureus* wall teichoic acid (WTA) – present in the old cell wall – acts as a repellent for the repeats, thereby directing the enzymes to the septum, where they bind to and are controlled by LTA (Schlag et al., 2010; Zoll et al., 2012). This suggests that cationic molecules such as RTDs and Pep5 bind to the polyanionic LTA, thereby liberate the enzymes such that they can degrade the cell wall in an uncontrolled manner. An antagonisation assay confirmed that LTA completely suppresses the antimicrobial activity of RTDs when added in a 4-fold molar excess in respect to the peptide (Table 12). In contrast, higher LTA concentrations (10-fold molar excess) are

necessary to antagonise the antibiotic action of Pep5 (Bierbaum and Sahl, 1985) which is consistent with the data obtained here (Table 12).

The number of positive charges of a given peptide might be of particular relevance for the interaction with LTA and thereby for the release of autolysins (Bierbaum and Sahl, 1987). A higher autolysin activity could be detected in the supernatant of cells exposed to RTD-2 (net charge +6) and Pep5 (net charge +7; which served as a positive control) compared to cells treated with RTD-1 (net charge +5).

Notably, the unprocessed Atl forms could also be detected in the supernatant of treated *S. aureus* cells (Figure 19), indicating that the processing of the enzyme occurs at the septum when bound to LTA.

Moreover, two mutants with a reduced LTA content (*S. aureus* SA113 Δ*ypfP* and *S. aureus* SEJ Δ*ltaS* (4S5)) were tested for their susceptibility towards RTDs and Pep5. Consistently, these strains have either a lowered autolysis rate (Fedtke et al., 2007) or a reduced amount of hydrolytic enzymes bound to the cell envelope (Corrigan et al., 2011). However, MIC determinations revealed that both strains were more susceptible to Pep5 (Table 11). Additionally, the LTA-deficient suppressor strain Δ*ltaS* (4S5) showed a decreased MIC value against RTDs compared to its parental strain (Table 11). As LTA are crucial for bacterial growth, the strain Δ*ltaS* (4S5) compensates the loss of LTA by increasing the intracellular level of the secondary messenger c-di-AMP. It has been suggested that this messenger plays a role in cell division, but it has to be further elucidated whether c-di-AMP is involved in the regulation of other cellular processes. *S. aureus* SEJ Δ*ltaS* (4S5) is also more susceptible to cell wall inhibitors such as nisin, vancomycin and penicillin as well as to daptomycin (Corrigan et al., 2011). Thus, the decreased MIC values of Pep5 and RTDs might be a secondary effect due to yet unknown altered cellular processes in this strain. Alternatively, it can be hypothesised that in case of a reduced LTA content, peptides more easily directly interfere with the bacterial membrane, thereby leading to membrane permeabilisation even in the absence of a high membrane potential.

In the strain *S. aureus* SA113 Δ*ypfP* the remaining LTA is anchored to the membrane via diacylglycerol (DAG) instead of diglucosyl-DAG. In this case, the binding of Pep5 to LTA could still have an impact on the membrane integrity as binding sites are even closer to the outside of the cytoplasmic membrane.

Interestingly, *S. simulans* and *S. carnosus* are more susceptible towards Pep5 (Sahl and Brandis, 1981) and θ-defensins than *S. aureus* (Table 10, Table 11). The LTA in these strains does not differ and consists of a polyglycerolphosphate chain linked to a glycolipid anchor (Ruhland and Fiedler, 1990). However, modifications of the LTA such as substitution of the glycerol units with D-alanine may have an impact on the susceptibility

as it adds a positive charge to the polyanionic molecules. Brötz et al. (1998) suggested that the Pep5 docking molecule might be more prominent or better accessible in these strains. Recently, a phylogenetic study of Atl proteins derived from 15 staphylococcal species revealed that the second repeat of the Atl-AM from *S. simulans* and *S. carnosus* completely differs from those of all other species (Albrecht et al., 2012). As Atl proteins target glycerophosphate-containing LTA via their repeats (Zoll et al., 2012), the sequence of the repeat unit might influence the binding to LTA. Thus, the enzymes might be easier released by cationic peptides such as RTDs and Pep5 in these species.

All the results described above indicate that Pep5 and RTDs may have a similar mode of action against staphylococci. This was further substantiated by the fact that the heterologous expression of PepI – which is secreted by the Pep5 producer strain to protect itself – provided partially cross-resistance towards RTDs (Table 13). However, this raises questions about the interaction partner for PepI. One could hypothesise that PepI forms a complex with LTA, thereby inhibiting the binding of the antimicrobial peptides and subsequently the displacement of the autolytic enzymes. Nevertheless, this interaction would probably also interfere with the targeting and the activity of Atl.

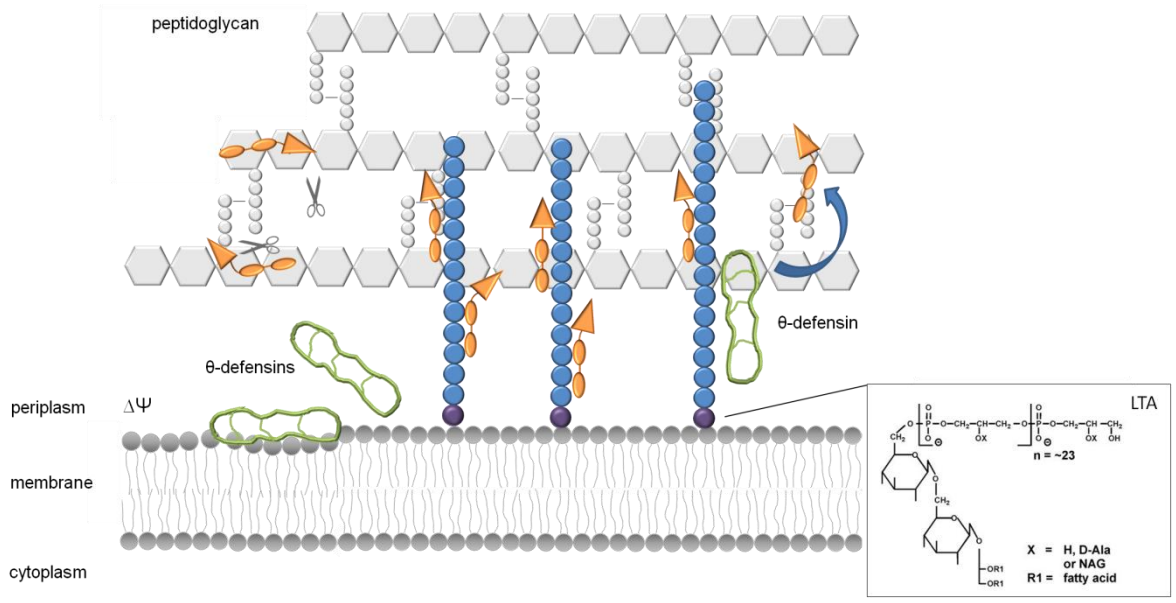

Figure 28 Proposed mode of action of θ-defensins. The peptides disrupt the membrane barrier function in presence of a high membrane potential (ΔΨ) by a yet unknown mechanism. Moreover, RTDs release autolytic enzymes (marked in orange) by interaction with LTA, thereby causing uncontrolled degradation of the cell wall.

In addition, RTDs exhibit activity against Gram-negative bacteria in a similar concentration range as against Gram-positives (Tang et al., 1999; Tran et al., 2008; Tran et al., 2002).

Tran et al. (2008) reported that RTD-1 and RTD-2 effectively permeabilise the outer and inner membrane of *E. coli* ML35-pYC, whereas RTD-3 (Figure 12) does not affect the membrane. This membrane impairment seems to be independent from the membrane potential as these assays were carried out in buffer lacking free glucose.
Further studies are necessary to analyse if RTDs additionally interfere with membrane bound molecules in Gram-negative bacteria.

4.3 Part 3: Interaction of hBD3 with the Gram-negative cell envelope

The antibacterial activity of the highly cationic peptide hBD3 was extensively studied in *S. aureus* and it has been demonstrated that binding to lipid II and subsequent inhibition of cell wall biosynthesis is a major determinant of its mechanism of action (Sass et al., 2008; Sass et al., 2010). Here, the interaction of hBD3 with the cell envelope of *E. coli* was investigated to gain a first insight into its antibiotic action against Gram-negative bacteria.
Initially, the defensin has to cross the outer membrane barrier of Gram-negative bacteria to gain access to the periplasmic space. This may occur by a mechanism known as self-promoted uptake which is based on the interaction of the cationic peptides with the negatively charged LPS molecules of the outer membrane (compare 1.3) (Hancock, 1997). In fact, hBD3 rapidly disrupted the outer membrane of *E. coli* ML35-pYC comparable to the membranolytic activity of polymyxin B (Figure 24). Consistently, evaginations of the outer membrane could be observed by transmission electron microscopy (Figure 25) which are indicative of LPS released from the cell surface (Spindler et al., 2011).
To further analyse the interaction of hBD3 with LPS, the susceptibility of strains with altered LPS structure was tested (Figure 26). LPS molecules typically consist of three distinct regions: a hydrophobic membrane anchor (lipid A), a short sugar chain substituted with phosphate groups (core) and a polymer composed of oligosaccharide repeats (O antigen). Interestingly, all three mutants of the *E. coli* K-12 strain BW25113 (Δ*waaP,* Δ*waaY,* Δ*waaG*) included in the study were more resistant towards hBD3. These mutants are affected in the assembly of the LPS core region. The WaaP and WaaY kinases are involved in the phosphorylation of the first and second heptose residue in the inner core (Yethon et al., 1998). Thus, a deletion of one of the genes reduces the net negative charge of the cell envelope which correlates with a decreased susceptibility towards cationic peptides, as observed here. On the other hand, the phosphoryl substituents are critical for the formation of a stable outer core, because their negative charge allows neighbouring LPS molecules to be crossbridged by divalent cations (Helander et al., 1989;

Yethon et al., 2000). It has been shown that *waaP* deletion mutants are highly susceptible towards polymyxin B or detergents such as SDS (Yethon et al., 2000; Yethon et al., 1998). Nevertheless, among the tested strains *E. coli* Δ*waaP* displayed the highest resistance.

The gene *waaG* encodes a glycosyltransferase which adds the first glucose to the outer core; consequently the deletion strain lacks the entire outer core region. These results suggest that not only the phosphate groups, but also the sugars of the outer core region may be crucial for the interaction with hBD3 whereas the O antigen does not seem to be involved in binding (Figure 26).

Further, the question is raised whether outer membrane permeabilisation is sufficient by itself to cause bacterial cell death or if additional target structures are involved in hBD3-mediated killing of Gram-negative bacteria.

It could be hypothesised that hBD3 interacts with the cell wall precursor lipid II once it enters the periplasmic space. Studies with chimeric and shortened hBD3 molecules linked the Gram-positive activity to the N-terminal part and the action against Gram-negative bacteria to the C-terminus (Hoover et al., 2003; Jung et al., 2011). This suggests that the highly charged C-terminal part of hBD3 (compare Figure 22) is essential for its interaction with the outer membrane, whereas the N-terminal part plays a predominant role in the interaction with lipid II. Moreover, hBD3 forms stable dimers in solution (due to an intermolecular salt bridge between E28 and K32 on another monomer) supporting the interaction with the membrane. Additionally, an intramolecular salt bridge involving the residues E27 and R17 (Schibli et al., 2002) contributes to stability and enhances the membrane-disrupting properties of hBD3 (Boniotto et al., 2003).

Consequently, hBD3 combines two activities in one molecule in contrast to oyster defensins which have only a weak membrane-disrupting activity and are barely active against Gram-negative bacteria. This correlates with the affinity to the cell wall precursor lipid II as discussed before (compare section 4.1).

However, growth kinetic measurements indicated that the mode of action of hBD3 against Gram-negative bacteria is different from the action against *S. aureus* (Figure 23) and that other target structures might also be involved in killing. Sass et al. (2010) demonstrated that the activity of hBD3 can be antagonised by the lipid II isoprenoid anchor C_{55}-P in a molar ratio of 2:1. This lipid carrier is also involved in the biosynthesis and modification of LPS, suggesting that hBD3 might also interfere with these processes. For example, the repeating units of the O antigen are assembled on C_{55}-P on the inner face of the cytoplasmic membrane. After transport across the membrane, the O antigen is polymerised and ligated to lipid A-core (Raetz and Whitfield, 2002; Wang and Quinn, 2010).

Further, the inner membrane was permeabilised to some degree by hBD3, but less efficiently as with polymyxin B (Figure 24), indicating a higher affinity of hBD3 to the outer membrane. Nevertheless, this interaction did not cause a significant loss of cytoplasmic content as monitored by transmission electron microscopy (Figure 25) and growth kinetic measurements (Figure 23). A similar lack of lytic effects has been reported for the synthetic antimicrobial peptide Bac8. This peptide was shown to cause partial membrane permeabilisation, disruption of the electron transport chain located in the inner membrane and cell depolarisation which in turn results in cell death (Spindler et al., 2011). Consistently, microarray data of hBD3 treated *S. aureus* cells indicated that energy generation is affected in presence of the peptide (Sass et al., 2008). This suggests that hBD3 might also inhibit cellular respiration by either direct or indirect disruption of respiratory functions.

In conclusion, binding to LPS and subsequent destabilisation of the cell envelope seems to play a significant role in the mode of action of hBD3 against Gram-negative bacteria and may be more important than the interaction with lipid II. Moreover, it is likely that hBD3 interferes with various membrane-bound processes in *E. coli* which may each contribute to the overall efficacy. Since the defensin causes stresses at many sites, it may act like “sand in the gearbox” – a mechanism which has been described for α-helical HDPs (Pag et al., 2008).

However, further studies are necessary to investigate the inhibition of these individual processes in more detail and to correlate them with the loss of cell viability (section 4.5).

4.4 Conclusion

Altogether, defensins of different groups interact with molecules of the microbial cell envelope that are directly accessible such as lipid II, LTA or LPS (Table 15). Thereby, the interaction between the defensin and the lipid-bound docking molecule can be highly specific as shown for lipid II and antibacterial CSαβ-type defensins.

Interestingly, similarities in the mode of action between some eukaryotic defensins and bacterially-produced lantibiotics could be revealed. The lantibiotic nisin and antibacterial invertebrate and fungal defensins bind to the pyrophosphate moiety of the highly conserved cell wall precursor lipid II. Moreover, Pep5 and θ-defensins do not only disrupt the membranes barrier function, but they also release the major autolysin Atl in staphylococci probably by replacing the enzymes from LTA.

Despite their low sequence similarity, these cationic peptides share many features as amphipathicity and cationicity. Moreover, their structures are stabilised by disulphide

bridges or (methyl)lanthionines, respectively. Thus, the antimicrobial activity may depend on the overall conformation and charge distribution rather than on their primary sequence. Since some defensins inhibit specific microbial targets without impacting membranes, they are considered attractive candidates for further development as anti-infective drugs. For instance, NZ2114 – an improved variant of the fungal defensin plectasin – showed potent activity in rodent infection models against clinically relevant strains such as MRSA and VRE, enhanced serum-stability, and extended *in vivo* half-life (Andes et al., 2009; Xiong et al., 2011).

Table 15 Known target structures of various defensin groups.

Defensin group	Example	Target structure	Reference
antibacterial CSαβ-type defensins	plectasin *Cg*-Defs	lipid II (high affinity)	Schneider et al., 2010 this study
antifungal CSαβ-type defensins	heliomicin Rs-AFP2	GlcCer	Thevissen et al., 2004 Aerts et al., 2009
α-defensins	HNP-1	lipid II (intermediate affinity)	de Leeuw et al., 2010
β-defensins	hBD3	lipid II (intermediate affinity) LPS	Sass et al., 2010 this study
θ-defensins	RTDs	LTA cytoplasmic membrane	this study

4.5 Outlook

In this study, the antistaphylococcal activity of two θ-defensins was investigated. It was shown that these peptides do not only impair the membrane barrier function, but also liberate the staphylococcal autolysin Atl from LTA in a similar way as the lantibiotic Pep5. However, several open questions remain to be further investigated. For example, MIC determinations revealed that the strains *S. aureus* SA113 Δ*ypf* and *S. aureus* SEJ Δ*ltaS* (4S5) – which are characterised by a reduced LTA content – were more sensitive towards Pep5 and in part to RTDs. In this context, it was reasoned that in absence of LTA the peptides can more easily interfere directly with the bacterial membrane. To confirm this hypothesis, the peptide-induced membrane perturbation of the mutants could be tested in comparison to the parental strain, e.g. by membrane potential measurements.

Moreover, it would be interesting to monitor the binding of the peptides to the bacterial cell envelope and the subsequent release of the enzymes using fluorescence microscopy. Zoll et al. (2012) demonstrated that externally applied Cy5-labelled repeats of the Atl-AM

accumulate at the cross wall of dividing *S. aureus* cells. Thus, it would be expected that treatment with RTDs causes a delocalisation of the repeats from the septum.
Further, it was shown that the heterologous expression of the immunity peptide PepI of the Pep5 producer strain provided partially cross-resistance towards RTDs. Nevertheless, further studies are necessary to gain a deeper insight into the mechanism by which the molecule confers resistance. Hoffmann et al. (2004) reported that PepI localises in the outer leaflet of the cytoplasmic membrane and that the highly charged C-terminal part is crucial for antagonising Pep5 activity. However, it is still unknown whether PepI forms a complex with lipid-bound molecules of the staphylococcal cell envelope or whether it interacts directly with the cationic peptides Pep5 or RTDs, thereby preventing interference with both the bacterial membrane and LTA. The fact that the C-terminus of PepI possesses several positively charged amino acids rather suggests that it forms a complex with anionic molecules such as LTA. Thus, it could be first investigated whether PepI can antagonise both activities of the peptides. The potassium efflux of energised cells expressing PepI under an IPTG-inducible promotor could be monitored to test whether PepI can inhibit the peptide-induced membrane permeabilisation (compare section 2.3.8). In additon, the supernatant of peptide treated cells expressing PepI could be analysed for cell wall lytic activity in comparison to control cells (compare section 2.5.3).

In the third part of this thesis, the interaction of hBD3 with the cell envelope of *E. coli* was investigated. It was demonstrated that the peptide permeabilise the outer membrane by binding to LPS and that the composition of the LPS core region plays an important role in this interaction. To gain a deeper insight into the interaction of LPS and hBD3, binding analysis using surface plasmon resonance could be carried out. Further, killing kinetics of hBD3 treated *E. coli* cells might correlate the permeabilisation of both membranes with the loss of cell viability.
Moreover, some interference of hBD3 with the inner membrane could be detected. In this context, it would be interesting to measure the depolarisation of the membrane in presence of hBD3 (compare section 2.3.9). Further, it has been suggested that hBD3 affects directly or indirectly the electron transport chain located in the inner membrane. To analyse the effect of hBD3 on respiration, the intracellular NAD^+/NADH ratios or the oxygen consumption of cells exposed to hBD3 should be measured. Specific inhibition of the individual electron transport chain complexes could be studied *in vitro* using inverted membrane vesicles.

5 Summary

Multicellular organisms defend themselves against infectious microorganisms by producing a wide array of antimicrobial peptides referred to as host defence peptides (HDPs). These evolutionary ancient peptides are important effector molecules of innate immunity. In addition to their immunomodulatory functions, they display potent direct antimicrobial activity against a broad range of microorganisms, including multiresistant pathogens. Thus, they are considered as promising candidates for the development of novel anti-infective agents.
Almost all HDPs are cationic and amphipathic. Hence, it has been generally assumed that these peptides interact unspecifically with negatively charged microbial membranes and subsequently disrupt membrane barrier functions. However, evidence is increasing that the antibiotic activity of HDPs can be much more targeted as it was originally proposed.
One important HDP class comprises defensins which are characterised by disulphide-stabilised β-sheets as the major structural component. In this work, the mode of action of defensins from diverse origins was investigated to gain further insights into the molecular mechanisms of defensin-mediated killing.

In the first part of this work, the antibacterial action of invertebrate defensins was studied using three defensin variants (*Cg*-Defm, *Cg*-Defh1 and *Cg*-Defh2) produced by the Pacific oyster *Crassostrea gigas*. These peptides exhibited potent activity against several Gram-positive bacteria, including staphylococci which were chosen for further mode of action studies. Interestingly, the oyster defensins did not compromise the membrane integrity since no impact on the membrane potential could be observed in cells exposed to these peptides. Instead, the defensins triggered the accumulation of the final soluble cell wall precursor UDP-MurNAc-pp in the cytoplasm, comparable to classical inhibitors of the membrane-bound steps of cell wall biosynthesis. Moreover, the oyster defensins were found to bind irreversibly to the cell wall precursor lipid II in a 1:1 stoichiometry, thereby causing sequestration of the precursor from penicillin binding proteins and subsequently blocking its further incorporation into the cell wall. Among the oyster defensins, *Cg*-Defh2 was the most potent, exhibiting 2- to 4-fold lower MIC values than *Cg*-Defh1 and *Cg*-Defh2, which correlated with different binding affinities for lipid II as revealed by surface plasmon resonance.

The second part of this thesis was aimed at elucidating the antistaphylococcal action of two rhesus macaque θ-defensins (RTD-1, RTD-2). These peptides differ structurally from all other defensins in that their backbones are cyclised by peptide bonds. Potassium efflux experiments and membrane potential measurements demonstrated that the membrane impairment by RTDs strongly depends on the energisation of the membrane. In addition,

RTD treatment caused the release of the cell wall lytic enzyme Atl probably by interaction with the membrane-bound lipoteichoic acid. Thus, the premature and uncontrolled activity of this enzyme contributes strongly to the overall killing by θ-defensins. Interestingly, a similar mode of action has been described for Pep5, an antimicrobial peptide of bacterial origin. Consistently, PepI – which is secreted by the Pep5 producer strain to protect itself – provided partially cross-resistance towards RTDs.

In the third part of this thesis, the antimicrobial activity of the human β-defensin 3 (hBD3) against Gram-negative bacteria was studied. Previous works have reported that binding to lipid II and subsequent inhibition of cell wall biosynthesis is a major determinant of its mechanism of action against staphylococci.
Here, it was demonstrated that the peptide permeabilises both the inner and outer membrane of *E. coli*. The interaction of the peptide with the lipopolysaccharides (LPS) of the outer membrane seems to play a significant role in the activity of hBD3 against Gram-negative bacteria and was shown to depend on the number of phosphate groups and the sugars in the LPS core region. Interestingly, the interaction with the inner membrane did not cause a significant loss of the cytoplasmic content as revealed by growth kinetic measurements and transmission electron microscopy of hBD3 treated *E. coli* cells. It remains to determine if additional target structures in the inner membrane such as the electron transport chain are also involved in hBD3-mediated killing.

In conclusion, previous reports and the results described in this thesis revealed that conserved molecules of the microbial cell envelope, which are readily accessible, are targets of various defensins and an important component of the killing mechanism. As demonstrated here for lipid II and oyster defensins, the interaction between the defensin and its lipid-bound target molecule can be highly specific.

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7 Publications

Journal articles

Sass, V., Schneider, T., **Wilmes, M.**, Körner, C., Tossi, A., Novikova, N., Shamova, O., and Sahl, H. G. (2010): Human beta-defensin 3 inhbitis cell wall biosynthesis in staphylococci. *Infect Immun* **78**, 2793-800

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Ulm, H., **Wilmes, M.**, Shai, Y., and Sahl, H. G. (2012): Antimicrobial host defensins – specific antibiotic activities and innate defense modulation. *Front Immunol* **3**: 249

Poster presentations

Wilmes, M., Tuchscherr, L., Becker, K., and Sahl, H. G.: Efficacy of human β-defensin 3 (hBD3) against clinical isolates of *Staphylococcus aureus*.
Annual conference of the German Society for Hygiene and Microbiology (DGHM), Göttingen, 2009

Wilmes, M., Schmitt, P., Schneider, T., Destoumieux-Garzón, D., and Sahl, H. G.: Mode of action of defensins against *Staphylococcus aureus*.
Transregio 34 Meeting on “Host pathogen interactions in bacterial infections”, Greifswald, 2010

Wilmes, M., Sass, V., Schneider, T., Tossi, A., and Sahl, H. G.: Mode of action of human β-defensin 3 (hBD3).
Annual conference of the Association for General and Applied Microbiology (VAAM), Karlsruhe, 2011

Wilmes, M., Sass, V., Schneider, T., Tossi, A., Ouellette, A., Selsted, M., and Sahl, H. G.: Comparison of the antistaphylococcal activity of human β-defensin 3 (hBD3) and rhesus macaque theta-defensins (RTD-1, RTD-2).
Gordon Research Conference on “Antimicrobial Peptides”, Lucca (Barga), Italy, 2011

Wilmes, M., Ouellette, A., Selsted, M., and Sahl, H. G.: Mode of action of rhesus macaque theta-defensins (RTD-1, RTD-2) against *Staphylococcus aureus*.
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Wilmes, M., Ouellette, A., Selsted, M., and Sahl, H. G.: Antistaphylococcal action of rhesus macaque theta-defensins.
Gordon Research Conference on “New Antibacterial Discovery and Development”, Lucca (Barga), Italy, 2012

8 Declaration (Eidesstattliche Erklärung)

Hiermit erkläre ich an Eides statt, dass ich für meine Promotion keine anderen als die angegebenen Hilfsmittel benutzt habe, und dass die inhaltlich und wörtlich aus anderen Werken entnommenen Stellen und Zitate als solche gekennzeichnet sind. Diese Arbeit ist weder identisch noch teilidentisch mit einer Arbeit, die an der Rheinischen Friedrich-Wilhelms-Universität Bonn oder einer anderen Hochschule zur Erlangung eines akademischen Grades oder als Prüfungsleistung vorgelegt worden ist. Teile der Dissertation sind vorab an den unter Punkt 7 „Publications“ aufgeführten Stellen auszugsweise veröffentlicht worden.

Bonn, Oktober 2012

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